PROPERTIES OF RIBONUCLEASE, PHOSPHODIESTERASE, AND DECYCLIZING PHOSPHODIESTERASE FROM RAPE (*Brassica napus* L.) POLLEN*

A.HOLÝ and M.PLŠEK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received September 12th, 1975

Three major enzymes of the EII protein isolated from bee-gathered rape pollen, namely, ribonuclease, phosphodiesterase, and decyclizing phosphodiesterase were examined by means of specific substrates. The pollen ribonuclease was characterised as nonspecific ribonucleate-2′-nucleotidyltransferase (EC 2.7.7.17) of a low hydrolytical activity. The enzyme hydrolyses RNA and diribonucleoside phosphates with the formation of ribonucleoside 2′,3′-cyclic phosphates. In the case of RNA, the pH optimum value is 5·5. The enzyme is relatively thermostable, does not require the presence of metal ions, and is inhibited by salts of bivalent heavy metals. The enzyme exclusively degrades the (3′ → 5′)-internucleotidic bonds of the D-*ribo* series with a 2′-hydroxylic function of the *ribo* configuration vicinal to the phosphodiester bond. The phosphodiesterase was characterised as ribonucleate-5′-nucleotidylhydrolase (EC 3·1·4) exclusively degrading internucleotidic bonds of the *all-ribo* configuration with the formation of 5′-ribonucleotides. The optimum pH value lies in the alkaline region and the enzyme requires the presence of a ribonucleoside attached to a 5′-nucleotide; it is nonspecific with respect to the heterocyclic base. The decyclizing phosphodiesterase splits specifically the ribonucleoside 2′,3′-cyclic phosphates to 2′-nucleotides and may be characterised as N-ribosylpurine(pyrimidine) 2′,3′-cyclic phosphate N-ribosylpurine(pyrimidine) 2′-phosphate hydrolase (EC 3.1.4d) nonspecific towards the nature of the heterocyclic base and similar to the spleen decyclizing phosphodiesterase. Its optimum pH value is 7·3, it is thermolabile and is inhibited by ions of bivalent heavy metals. As indicated by kinetic data, the base does not participate in the hydrolytical process, its engagement in the formation of the substrate being of a cooperative character only.

As reported in the preceding paper[1], two nucleolytically active protein fractions have been isolated from the bee-gathered pollen of rape (*Brassica napus* L.). One of them, the EII protein fraction contains three nucleolytic activities, namely, ribonuclease, phosphodiesterase, and decyclizing 2′,3′-phosphodiesterase while 3′-nucleotidase free of any further contaminating activities was present in the EIII protein fraction. All these four enzymes may be separately examined with the use of selected substrates. In connection with investigations on specificity of nucleolytic enzymes

* Presented in part on the IInd Symposium on Ribosomes and RNA Metabolism, Smolenice Castle, Slovakia, Czechoslovakia, September 1975. Taken in part from the Thesis of M.P. (submitted to Charles University, Prague).

and particularly on properties of analogous enzymes from various sources[2-6], it appeared of interest to gain information on properties and substrate specificity of the present enzymes. The aim of the work shown in this paper was to characterise properties and structural requirements of these enzymes and to compare them with data on analogous enzymes of another origin.

Rape Pollen Ribonuclease

The EII protein fraction from the extract of bee-gathered rape pollen has been shown in the preceding paper[1] to contain a ribonuclease which is nonspecific to the nature of the heterocyclic base of the nucleotide. The hydrolytic activity is suppressed since the enzyme catalyses mainly the transfer reaction (transfer of the 3'-ribonucleotide residue to the vicinal 2'-hydroxylic function and formation of a ribonucleoside 2',3'-cyclic phosphate). Because of the simultaneous presence of decyclizing phosphodiesterase, it was not possible to examine directly the stability of 2',3'-cyclic phosphates towards the EII protein ribonuclease. In the region of acidic pH values, the ribonuclease is the sole enzyme capable of degrading RNA (*cf.*[1]). Consequently, the properties of the enzyme can be preferably examined in the acidic region.

The optimum pH value in hydrolysis of RNA with ribonuclease was determined by measurements of the initial rate of this hydrolysis under conditions of the Kunitz assay. As it may be seen from Fig. 1, the optimum pH value is about 5·5. Like other ribonucleases, the enzyme is relatively thermostable. Thus, the original activity decreases only by 30% when the enzyme is exposed to the temperature of 60°C for 20 min at pH 5·5 (Table I). The pollen ribonuclease does not require the presence

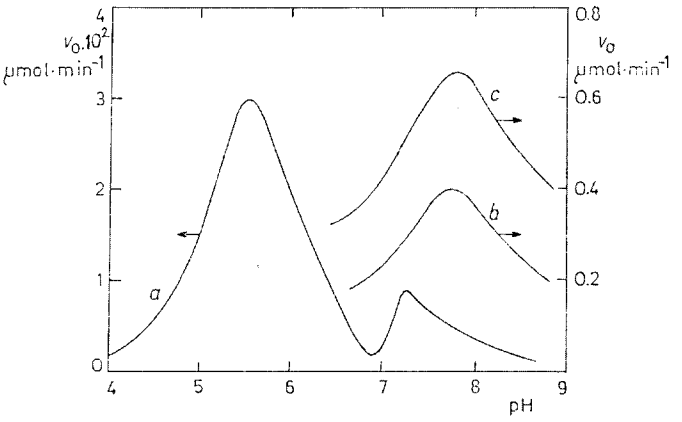


FIG. 1

pH-Dependence of the Initial Rate in Degradations with the EII Protein Fraction
a RNA, *b* Ucp, *c* Acp.

of metal ions. The presence of ethylenediamine tetraacetic acid does not affect the activity of the enzyme towards RNA (Table II); in the presence of magnesium ions, the activity slightly decreases while the heavy metal ions such as Fe^{2+} and particularly Zn^{2+} and Cu^{2+} completely inhibit the activity of the enzyme. Such a sensitivity towards heavy metal ions may be regarded as a general property of ribonucleases[7].

The nonspecificity of pollen ribonuclease towards the heterocyclic base of the substrate as observed[1] in degradation of RNA, was confirmed by degradation of diribonucleoside phosphates containing all the four naturally occurring nucleosides both as the first and the second unit of the doublet (compounds *I*–*IX*; see also Table III). An exact evaluation of the relative extent of the degradation in these combinations is hardly possible since the tests were performed (for reasons see below) at pH 7·3, *i.e.*, at such a pH value when the activity of the accompanying phosphodiesterase asserts itself. Nevertheless, degradation of dinucleoside phosphates *I*–*IX* with ribonuclease is clearly confirmed by analysis of degradation products. Neither the isomeric (2′ → 5′)-diribonucleoside phosphate *XV* nor the (5′ → 5′)-isomer *XVI*

TABLE I

Thermic Deactivation of Rape Pollen Ribonuclease and Decyclizing Phosphodiesterase at 60°C

Time, min	0	2	5	10	20
V_0[a] Ribonuclease[b]	1·00	1·00	1·00	0·86	0·72
V_0[a] Decyclizing phosphodiesterase[c]	1·00	0·94	0·62	0·62	0·42

[a] Initial velocity referred to the non-treated enzyme; [b] for RNA at pH 5·5 and 25°C; [c] for uridine 2′,3′ cyclic phosphate at pH 7·3 and 37°C.

TABLE II

Effect of Salts on the Activity of Rape Pollen Ribonuclease and Decyclizing Phosphodiesterase

Compound	Concentration	V_0[a] ribonuclease[b]	V_0[a] Decyclizing phosphodiesterase[c]
$MgSO_4$	10^{-2}	0·84	1·05
$FeSO_4$	10^{-2}	0·22	0·62
$CuSO_4$	10^{-3}	0	0·28
$ZnSO_4$	10^{-3}	0	0·60
NaF	10^{-2}	1·00	1·01
EDTA	10^{-2}	1·01	1·00

[a–c] *cf.* Table I.

are degraded by rape pollen ribonuclease in full accordance with specificity of ribonucleases towards (3′ → 5′)-internucleotidic bonds. Furthermore, the degradation does not occur with analogues containing at position 2′ either a substituted hydroxylic function (*XI*, *XII*) or other atoms instead of the hydroxylic group (*X*, *XIII*) and in the case of the *arabino* analogue *XIV* with a reversed configuration of the hydroxylic function mentioned. All these cases exclude formation of an activated cyclic intermediate as a *conditio sine qua non* for the conversion of an internucleotidic bond into a 2′,3′-cyclic phosphate.

TABLE III

Degradation of Dinucleoside Monophosphates with Rape Pollen Ribonuclease and Phosphodiesterase at pH 7·3

No	Compound[a]	Degradation, %	Product isolated[b]
I	UpU	78	Up, Urd; (pU) Ccp
II	UpC	73	Up, Cyd; (Urd, pC)
III	UpA	100	Up, Ucp, Ado; (pA, Urd)
IV	UpG	68	Up, Guo;, (Urd, pG)
V	CpC	100	C-2′-p; Ccp, Cyd (pC)
VI	ApU	100	A-2′-p; Urd (Ado, pU)
VII	ApA	85	A-2′-p, Ado (pA)
VIII	GpC	78	G-2′-p, Cyd (Guo, pC)
IX	GpG	87	G-2′-p, Guo (pG)
X	d(UpC)	10	dUrd, pdC
XI	$U_p^{THP}U$	0	—
XII	$U_p^{OMe}U$	0	—
XIII	2′-CldUpU	0	—
XIV	araCpU	0	—
XV	(2′ → 5′)-UpA	0	—
XVI	(5′ → 5′)-UpU	0	—

[a] Abbreviations: one-letter symbolics with nucleotides and dinucleoside phosphates, three-letter symbolics with nucleosides; internucleotidic linkage is of (3′ → 5′) type. Xcp, 2′,3′-cyclic phosphate; pX, 5′-nucleotide; Xp, 2′(3′)-nucleotide; X-2′-p, 2′-nucleotide; $U_p^{THP}U$, 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-uridine; $U_p^{OMe}U$, 2′-O-methyluridylyl-(3′ → 5′)-uridine; 2′-CldUpU, 2′-chloro-2′-deoxyuridylyl-(3′ → 5′)-uridine; araCpU, uridylyl-(5′ → 3′)-1-(β-D-arabinofuranosyl)-cytosine; [b] compounds in parentheses correspond to the action of phosphodiesterase.

The rape pollen ribonuclease thus may be designated as nonspecific ribonucleate-2′-nucleotidyltransferase (cyclizing) (EC 2.7.7.17) of a low hydrolytic activity and producing ribonucleoside 2′,3′-cyclic phosphates by degradation of internucleotidic bonds. Enzymes of this type are known in the field of both microorganisms (*B. subtilis* RNase[8]) and plants[9,10]. Ribonuclease of *Cycas revoluta* pollen exhibits similar properties, *i.e.*, it degrades nonspecifically RNA to ribonucleoside 2′,3′-cyclic phosphates and its pH optimum value is 5·7 (*cf.*[11]).

Rape Pollen Phosphodiesterase

The presence of this enzyme in the EII protein fraction of rape pollen has been established indirectly by analysis of products formed by degradation of dinucleoside phosphates with the EII protein fraction; this enzyme manifests itself exclusively in alkaline and neutral pH region. Because of the simultaneous presence of ribonuclease and the low level in the mixture, the information on rape pollen phosphodiesterase with respect to its stability and effect of ions is hardly accessible. The optimum pH value of this enzyme lies in the alkaline region.

Degradation of internucleotidic bonds of the (3′ → 5′)-type with rape pollen phosphodiesterase affords 5′-ribonucleotides (Table III; *cf.*[1]). The action of this rape pollen enzyme is thus similar to that of the snake venom phosphodiesterase[12]. It may be seen from Table III that rape pollen phosphodiesterase degrades the natural internucleotidic bonds regardless the character of the base in the nucleoside at the 5′- or 3′-end (compounds *I*–*IX*) but does not degrade the isomeric (2′ → 5′)- and (5′ → 5′)-bonds (compounds *XV* and *XVI*); the latter isomers (as derivatives of 5′-nucleotides) are split by the snake venom phosphodiesterase. Another difference between the two enzymes consists in affinity to compounds *XI*–*XIV* which represent the esters of 5′-ribonucleotides with a nucleoside different from ribonucleoside. In the series mentioned, the 2′-hydroxylic function vicinal to the 3′-phosphate bond was systematically modified. In all cases examined, this modification results in a complete loss of affinity to rape pollen phosphodiesterase. On the other hand, these substrate analogues are well degraded by the snake venom phosphodiesterase. It may thus be inferred from these observations that the rape pollen phosphodiesterase requires the presence of a ribonucleoside at the 5′-end of the molecule. The resistance of cytidine 5′-phosphate methyl ester (*XIX*, Table IV) towards the EII protein fraction may be regarded as a direct proof of this hypothesis. As an ester of a 5′-ribonucleoside, compound *XIX* ought to be subjected to the degradation; however, the reaction does not take place because of the absence of the corresponding ribonucleoside unit at the 5′-end of the substrate molecule.

On the basis of the above substrate requirements, the rape pollen phosphodiesterase may be classified as ribonucleate-5′-nucleotidohydrolase of the EC 3.1.4 series, nonspecific towards the heterocyclic base. Only a few enzymes of this type have been

reported in the literature such as exonuclease *E. coli*[13] from the group of RNA-specific exonucleases, or, ribonuclease of guinea pig liver nuclei[14], ribonuclease of the *Naja oxiana*[15] venom, ribonuclease of rat liver mitochondria[16], and finally ribonuclease M2 of *Phaseolus aureus* ROXB.[17], all from the group of the so called ribonucleases-hydrolases producing 5'-ribonucleotides. As the sole from the above group, the *Phaseolus aureus* enzyme is capable of producing 5'-mononucleotides (by the other above enzymes, RNA is degraded to oligonucleotides with a 5'-end phosphate only); this enzyme however, is activated by zinc ions. An analogous enzyme is apparently present in seeds of *Lolium multiflorum* L.[18]

Rape Pollen Decyclizing Phosphodiesterase

The decyclizing (cyclic) 2',3'-phosphodiesterase[1] is another enzyme present in the EII protein fraction. This enzyme is responsible for the presence of 2'-ribonucleotides in the RNA hydrolysate produced by the EII fraction: the previously formed (by the action of ribonuclease) ribonucleoside 2',3'-cyclic phosphates are hydrolysed under catalysis of decyclizing phosphodiesterase to isomer-free 2'-ribonucleotides. In the plant material[19-21], some phosphodiesterases have been detected which catalyse the hydrolysis of ribonucleoside 2',3'-cyclic phosphates to 3'-ribonucleotides. Only a few cyclic phosphodiesterases are known (such as the calf spleen decyclizing phosphodiesterase[2]) that are capable of converting the 2',3'-cyclic phosphodiesters into 2'-ribonucleotides. Since these enzymes hydrolyse a single type of substrates, namely, ribonucleoside 2',3'-cyclic phosphates, and the ribonuclease simultaneously present in the EII protein fraction does not attack the 2',3'-cyclic phosphates, it is possible to examine the properties and specificity of rape pollen decyclizing phosphodiesterase with the use of 2',3'-cyclic nucleotides as substrates. The enzyme was characterised by means of uridine 2',3'cyclic phosphate. The optimum pH value of this enzyme is pH 7·3 (Fig. 1). Ions of heavy metals such as Zn^{2+}, Cu^{2+}, and Fe^{2+} act as strong

TABLE IV
Degradation of Ribonucleotide Methyl Esters with Protein EII Rape Pollen

No	Ribonucleotide	Degradation, %	
		3 h	24 h
XVII	Cytidine 2'-phosphate	0	0[a]
XVIII	Cytidine 3'-phosphate	30	50[a]
XIX	Cytidine 5'-phosphate	0	0[a]
XX	Uridine 3'-phosphate	100[a]	—

[a] Checked by paper electrophoresis at pH 7·5.

inhibitors whereas the presence of fluoride anions, magnesium cations or of EDTA does not affect the degradation rate at all (Table II). Contrary to the rape pollen ribonuclease (*vide supra*), the pollen decyclizing phosphodiesterase is strongly thermolabile; it is deactivated at 60°C even in the neutral pH region (Table I).

As inferred from measurements on four "natural" 2',3'-cyclic nucleotides, the hydrolytical reaction corresponds to conditions of simple Michaelis kinetics. Inhibition of the enzyme by the product does not assert itself in kinetics under the experimental conditions. Characteristic kinetic data are shown in Table V. As indicated by K_m data, the affinity of all the four structurally different substrates towards the enzyme is approximately the same; the K_m values are constant within one order of magnitude and roughly correspond to data published in the case of other decyclizing phosphodiesterases[22]. As the main factor determining the stability of the ES-complex that portion of the molecule may be regarded which is in common to all the four substrates, namely, the ribofuranoside 2',3'-cyclic phosphate. In this moiety, the cyclic phosphodiester group dissociated to the 1st degree is oriented *trans* with respect to the heterocyclic base. This base is obviously of some importance in the complex formation (the affinity of the two pyrimidine derivatives is higher than that of the purine derivatives) but its role might be of a cooperative character. The enzyme is evidently nonspecific with respect to the base which does not therefore play any role in the binding site of the enzyme. The maximum initial rate (V_{max}) is somewhat more dependent upon the character of the heterocyclic base than the K_m value (Table V) but the difference varies within one order of magnitude. It is thus hardly probable that some of the functional groups typical of the four bases (uracil, cytosine, adenine, and guanine) could directly participate in the catalytical process.

Since the rape pollen cyclic phosphodiesterase resembles the spleen cyclic phosphodiesterase, it was of interest to compare the substrate specificity of the two enzymes. The comparison was performed with the use of a set of five-membered cyclic phosphodiesters derived from nucleoside analogues with modified sugar or heterocyclic

TABLE V

Michaelis Constants for Rape Pollen Decyclizing Phosphodiesterase Induced Hydrolysis of Ribonucleoside 2',3'-Cyclic Phosphates

Ribonucleoside	$K_m \cdot 10^{-2}$, M	$V_{max} \cdot 10^{-6}$, mol min^{-1}
Uridine	4·76	7·77
Cytidine	2·46	2·82
Adenosine	7·15	7·80
Guanosine	7·40	4·88

TABLE VI

Rape Pollen Decyclizing Phosphodiesterase Degradation of Nucleoside 2′,3′-Cyclic Phosphates

No	Ribonucleoside	Splitting, %		Spleen cPDase[a]
		3 h	24 h	
XXI	uridine	80	100	++
XXII	5-ethyluridine	59	100	++
XXIII	5-chlorouridine	54	100	++
XXIV	5-bromouridine	53	100	–
XXV	5-aminouridine	65	100	++
XXVI	5-dimethylaminouridine	26	71	+
XXVII	6-methyluridine	14	51	–
XXVIII	N^3-methyluridine	15	47	+
XXIX	cytidine	100	100	++
XXX	isocytidine	25	56	+
XXXI	5-methylcytidine	16	71	+
XXXII	6-azauridine	0	40	+
XXXIII	6-azacytidine	10	60	+
XXXIV	1-(β-D-ribofuranosyl)-4-methyl-2-pyrimidinone	56	100	++
XXXV	adenosine	81	100	++
XXXVI	N^6-dimethyladenosine	46	83	+
XXXVII	2-hydroxyadenosine	45	60	++
XXXVIII	inosine	33	81	++
XXXIX	6-thioinosine	9	20	+
XL	guanosine	55	100	++
XLI	8-bromoguanosine	35	62	+
XLII	8-methylguanosine	21	44	–
XLIII	8-thioguanosine	0	0	–
XLIV	xanthosine	68	100	++
XLV	α-uridine	10	62	–
XLVI	L-cytidine	0	0	–
XLVII	L-adenosine	18	46	+
XLVIII	9-(α-D-lyxofuranosyl)adenine	20	46	+
XLIX	1-(β-D-ribopyranosyl)uracil[b]	41	48[c]	–
L	1-(2,3-dihydroxypropyl)uracil	0	0	–

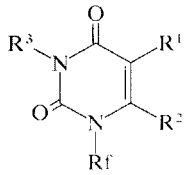
[a] ++ good substrate, + weak substrate, – non-substrate; [b] mixture with 3′,4′-phosphate; [c] no additional degradation on isolation and repeated enzyme treatment.

moieties. Since the great number of test substances does not permit to perform detailed kinetic measurements, the earlier reported[2-5] technique was used and the extent of degradation was examined in two time intervals under identical conditions (at a selected pH optimum value). The experimental values shown in Table VI were corrected with respect to the nonenzymatic hydrolysis. The observations are discussed in paragraphs *a*) to *d*).

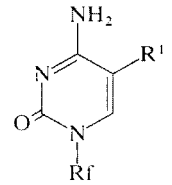
a) The enzyme exhibits a low specificity towards the modification of the heterocyclic base in the substrate. In a series of pyrimidine derivatives with modified uracil (compounds *XXI*–*XXVIII*) or cytosine (compounds *XXIX*–*XXXI*) systems, all compounds are degraded regardless the substituent or its position. Only the presence of a 6-methyl group results in a decreased extent of the degradation (a changed conformation of the molecule or a decreased occurrence of that conformation which would be suitable for the cooperative interaction of the base with the protein[23]). Replacement of the oxo or amino group at position 4 by a methyl group (*cf.* the derivative *XXXIII*) or a change in the π-electron system of the base and in polarisability of functional groups in comparison with the uracil system (*cf.* compounds *XXXI* and *XXXIII*) do not negatively affect the degradation. Only the replacement of uracil by its 6-aza analogue results in a decreased degradation (similar to the spleen cyclic phosphodiesterase); responsibility for this effect may also be ascribed to an increased acidity of the substrate due to a shift of the pK_a value of the heterocyclic base into the acidic region, and an increased probability of untypical interactions with basic parts of the protein at the expense of the genuine ES-complex formation.

The substrate specificity is also low in the series of purine derivatives. Thus, the degradation is almost unaffected by the character and mutual position of substituents (H, NH_2, OH) at position 2 and 6 of the purine ring (compounds *XXXV* to *XXXVIII*, *XL*, and *XLIV*) or by substitution at position 8 of the imidazole ring (compounds *XLI* and *XLII*) which might play some role in the conformation of the substrate[3]. The degradation rate is clearly lower, but the derivatives are substrates. Only the introduction of the thioxo group results in a strong inhibition of the degradation (compounds *XXXIX* and *XLIII*). Since this effect does not depend on the position of the substituent it may be assumed that the present inhibition is due to the known general interference of C=S or =C—SH groups of the substrate with the protein which is not in direct connection with the substrate requirements of the enzyme.

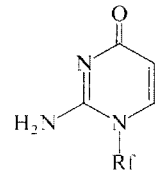
b) The group of substrates containing a modified sugar moiety supports the idea of a complementary effect to the effect of the heterocyclic base in the substrate–enzyme interaction. Thus, in degradation of the α-anomer *XVL* such a cooperative effect cannot assert itself; hence the markedly lowered degradation rate of the analogue *XLV* when compared with that of the parent uridine 2′,3′-cyclic phosphate (*XXI*). A similar marked contrast to degradation of the natural cyclic nucleotides may be



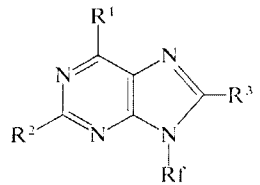
XXI, $R^1 = R^2 = R^3 = H$
XXII, $R^1 = CH_3$, $R^2 = R^3 = H$
XXIII, $R^1 = Cl$, $R^2 = R^3 = H$
XXIV, $R^1 = Br$, $R^2 = R^3 = H$
XXV, $R^1 = NH_2$, $R^2 = R^3 = H$
XXVI, $R^1 = N(CH_3)_2$, $R^2 = R^3 = H$
XXVII, $R^1 = R^3 = H$, $R^2 = CH_3$
XXVIII, $R^1 = R^2 = H$, $R^3 = CH_3$



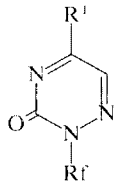
XXIX, $R^1 = H$
XXXI, $R^1 = CH_3$



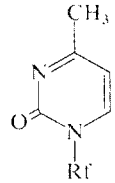
XXX



XXXV, $R^1 = NH_2$, $R^2 = R^3 = H$
XXXVI, $R^1 = N(CH_3)_2$, $R^2 = R^3 = H$
XXXVII, $R^1 = NH_2$, $R^2 = OH$, $R^3 = H$
XXXVIII, $R^1 = OH$, $R^2 = R^3 = H$
XXXIX, $R^1 = SH$, $R^2 = R^3 = H$
XL, $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$
XLI, $R^1 = OH$, $R^2 = NH_2$, $R^3 = Br$
XLII, $R^1 = OH$, $R^2 = NH_2$, $R^3 = CH_3$
XLIII, $R^1 = OH$, $R^2 = NH_2$, $R^3 = SH$
XLIV, $R^1 = R^2 = OH$, $R^3 = H$

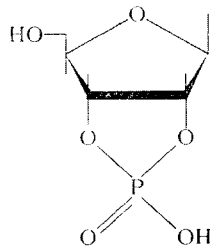


XXXII, $R^1 = OH$
XXXIII, $R^1 = NH_2$



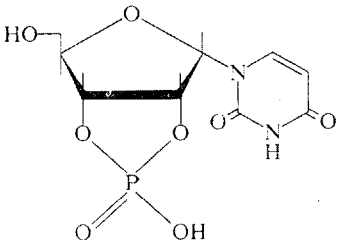
XXXIV

In formulae *XXI*—*XLIV* Rf =

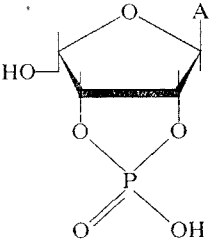


observed in the case of the L-enantiomers *XLVI* and *XLVII*. These analogues which do not chemically differ from the natural substrates, are bound to the enzyme by mediation of the phosphodiester group of the sugar moiety while the heterocyclic base being placed in another site of the enzyme cannot interact in a suitable manner. In analogy to the spleen cyclic phosphodiesterase, this effect asserts itself particularly

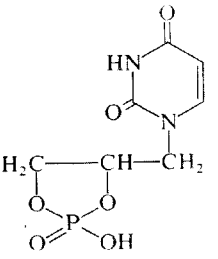
with pyrimidine derivatives. The adenine derivative *XLVII* is degraded since its purine π-electron system is larger than the π-electron system of the pyrimidine ring and is thus able to mediate, at least partially, the interaction between the base and the enzyme[2].



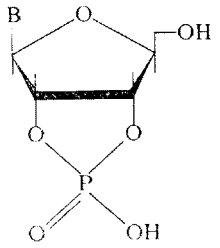
XLV



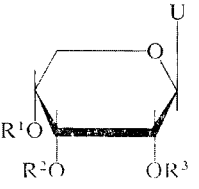
XLVIII
A = adenin-9-yl

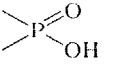


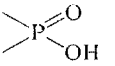
L



XLVI, B = cytosin-1-yl
XLVII, B = adenin-9-yl



XLIXa, R^1 = H, R^2, R^3 = 

XLIXb, R^3 = H, R^1, R^2 = 

U = uracil-1-yl

The change of configuration on the carbon atom 4′ results in a weakly negative effect on the degradation as exemplified by the lyxofuranosyl derivative *XLVIII*. Since the hydroxymethyl group (oriented *cis* with respect to the phosphate) can hardly intervene in the formation of a complex with the enzyme, the degradation of compound *XLVIII* could be more likely influenced by polar or hydrophilic effects on the active centre of the enzyme or by distribution of the phosphorus atom electrons. A deeper modification of the sugar-phosphate portion of the molecule such as replacement by an isomeric aliphatic chain (compound *L*) or enlargement of the phosphodiester bond (the six-membered 3′,5′-cyclic phosphate *LI*) usually results in loss of affinity towards the enzyme. The resistance of compound *LI* simultaneously confirms the absence of any 3′,5′-cyclic phosphodiesterase in the enzyme preparation.

In the uridine 2′,3′-cyclic phosphate isomer *XLIX*, the five-membered ribofuranose ring is replaced by the six-membered ribopyranose ring and the hydroxylic

functions (of a secondary alcohol character) are in *cis* configuration; furthermore, the specimen represented a mixture of 2',3'- and 3',4'-cyclic phosphates *XLIXa,b*. In the presence of the pollen cyclic phosphodiesterase, a 50% degradation of this specimen may be observed, the remainder being resistant to a further action of the enzyme. Most probably, only the 2',3'-cyclic phosphate *XLIXa* undergoes the enzymatic hydrolysis. The enzymatic degradation affords as product a monophosphate (either a 2'-phosphate or a 4'-phosphate) which is capable of forming a borate complex. Analogously to the ribonucleoside derivatives, the monophosphate is assumed to be a 2'-isomer. The rape pollen cyclic phosphodiesterase is thus the only enzyme which has been observed to degrade the cyclic nucleotides containing the pyranose ring[24].

c) Degradation of ribonucleoside 2',3'-cyclic phosphates with cyclic phosphodiesterase affords 2'-nucleotides as products. This conclusion was confirmed by analysis of products obtained by degradation of 2',3'-cyclic phosphates of cytidine, adenosine and guanosine in suitable systems[25,26]. The 2'-isomer has been detected as degradation product both of the lyxofuranosyl derivative *XLVIII* and the L-adenosine derivative *XLVII*. The stereospecificity of the ring opening thus asserts itself also in the case of the enantiomer; the results are in accordance with the conception on the 2',3'-cyclic phosphate group bound to the ribofuranosyl residue, with the immaterial effect of the 4'-hydroxymethyl group (*vide supra*), and is confirmed by comparison of structures *XXXV* and *XLVII*. The substituents at positions 1' and 4' are interchangeable; activation of the phosphodiester is obviously symmetrical with respect to the plane of symmetry of the sugar ring and the dynamic stereochemistry of the activated state must be determined by distribution of π-electrons on the sugar ring. Since the distribution is in both enantiomers affected by the heterocyclic base to an equal degree, a 2'-nucleotide is formed in both cases (ref.[2]).

d) The cyclic phosphodiesterase of rape pollen is much less dependent upon modifications of the substrate such as substitution or other changes on the heterocyclic base on the one hand or changes in the stereochemistry of the sugar ring on the other hand, than the enzyme from spleen[2]. The observed differences (compounds *XXIV*, *XXVII*, and *XLI*, or *XLV*) can be ascribed to a relatively higher participation of the base on the stabilisation of the ES-complex in the case of the spleen cyclic phosphodiesterase and the more pronounced effect of conformational variations of the whole molecule and structural variations of the base. It may be assumed *vice versa* that forces included in the binding or catalytic site which determine the formation of the complex (or the course of its reaction) are stronger in the case of the pollen cyclic phosphodiesterase; consequently, the increment of cooperative factors is lower. On the basis of the present observations, the rape pollen cyclic phosphodiesterase may be classified as N-ribosylpurine(pyrimidine) 2',3'-cyclic phosphate: N-ribosylpurine(pyrimidine) 2'-phosphate hydrolase (EC 3.1.4.d). The above com-

parison of various requirements of 2′,3′-cyclic phosphodiesterases from pollen and from spleen may be considered as a further confirmation of the earlier observations[3-6] that enzymes of similar characters and purposes, though isolated from various evolutionary remote species, usually differ little in their structural requirements.

EXPERIMENTAL

Methods and Materials

Paper chromatography was performed by the descending technique on paper Whatman No 3 MM in solvent systems S_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2); S_2, saturated aqueous ammonium sulfate–1M sodium acetate–2-propanol (79 : 19 : 2); and S_3, 2-propanol–0·1M triethylammonium borate (pH 7·5)–conc. aqueous ammonia (7 : 2 : 1). Paper electrophoresis was performed by the technique of Markham and Smith[27] at 20 V/cm for 1 h on paper Whatman No 3 MM in the buffer solution E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5). Spectrophotometric measurements were performed in aqueous solutions at 260 nm on a Spectromom 203. All the substrates examined in this work have been prepared earlier by reported[2] methods. The substances were used as sodium, lithium or ammonium salts and their content was determined previously by spectrophotometry. The RNA in kinetic measurements and activity assays was a gift of Dr G. S. Ivanova (Pushchino on Oka, U.S.S.R.). The activity of enzymes towards RNA was tested by the modified method of Kunitz (*cf.*[1]).

Degradations with the Rape Pollen EII Protein Fraction

Degradation of diribonucleoside phosphates. The incubation mixture contained 2 μmol of the appropriate substrate (ammonium salt) and 200 μg of the EII protein fraction in 100 μl of 0·05M TRIS-HCl buffer solution (pH 7·5) with 0·1M-NaCl. After the incubation at 37°C for 3 h and 24 h, 50 μl aliquots of the mixture were analysed by chromatography in the solvent system S_1. The spots of the substrate and degradation products were eluted with water (10 ml each) and the eluates subjected to spectrophotometric measurements. For the results see Table III.

Degradation of ribonucleoside 2′,3′-cyclic phosphates. The incubation mixture contained 3 μmol of the appropriate substrate (ammonium salt) and 165 μg of the EII protein fraction in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·3). After the incubation at 37°C for 3 h and 24 h, 50 μl aliquots were withdrawn and chromatographed in the solvent system S_1. Blanks were processed similarly but in the absence of the enzyme. Spots of starting compounds and degradation products were eluted with water and the eluates subjected to spectrophotometric measurements at 260 nm. For the results see Table III.

Degradation of nucleoside alkyl esters. The appropriate substrate (3 μmol) in 100 μl of 0·05M TRIS-HCl buffer solution (pH 7·3) was incubated at 37°C in the presence of the EII protein fraction (165 μg). Aliquots (50 μl each) were withdrawn after 3 h and 24 h and chromatographed in the solvent system S_1. The presence of a free nucleotide has not been detected in any case. Since the system S_1 does not guarantee separation of the starting substance from a nucleoside which could be formed by dephosphorylation of the product, the spots corresponding to the starting substance were eluted with a small volume of water and the eluate analysed by electrophoresis in the buffer solution E_1. For the results see Table IV.

Identification of Ribonucleoside 2′-, 3′-, and 5′-Phosphates

The degradation of the substrate was performed according to the above general procedure. The spots of nucleotides were eluted with a minimum volume of water and the eluates were freeze-dried. The presence of 5′-ribonucleotides was demonstrated by rechromatography of the eluate in the solvent system S_3; the isomeric 2′- and 3′-ribonucleotides were separated by chromatography in the solvent system S_2 (derivatives of adenosine and guanosine) or on a column of Dowex 1 (10 × 1 cm) (formate cycle) ion exchange resin in 0·02M formic acid in the case of cytidine nucleotides [1].

Determination of K_m and V_{max} Values for the Cyclic Phosphodiesterase of EII Protein Fraction

The incubation mixture contained the appropriate substrate ($1 . 10^{-2}$M to $6 . 10^{-1}$M) and the EII protein fraction (30—80 μg) in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·8). On the basis of preliminary experiments the degradation was performed at 37°C for 45 min with such an amount of the enzyme (protein) that the extent of the degradation did not exceed 30%. Thus, after 45 min at 37°C, the incubation mixture was chromatographed in the solvent system S_1 and the content of spot eluates determined spectrophotometrically at 260 nm as above. Concentrations of substrates were also determined spectrophotometrically (an aliquot of the stock solution of the substrate was diluted with 0·01M-HCl and the A_{260} was measured). In calculations, tabulated extinction coefficient values were used[28]. For kinetic data see Table V.

pH-Dependence of Degradation Rate in the EII Protein Fraction Assays

The incubation mixture consisted of 3 μmol of uridine 2′,3′-cyclic phosphate (lithium salt) or adenosine 2′,3′-cyclic phosphate (ammonium salt), the EII protein fraction (250 μg), and 100 μl of the appropriate 0·05M buffer solution containing 0·2M-NaCl. After 1 h at 37°C, an aliquot of the incubation mixture was chromatographed in the solvent system S_1, the spots of the starting material and degradation products eluted, and the content of eluates determined spectrophotometrically as above. Degradation of RNA (2 mg) was performed with 100 μg of the EII protein fraction in 650 μl of the appropriate buffer solution containing 0·2M-NaCl (25 min at 25°C). The incubation was stopped by the addition of 0·75% uranyl acetate in 25% aqueous perchloric acid (250 μl). The mixture was then diluted with water (600 μl), kept at 0°C for 2 h, filtered, the filtrate diluted with 300 parts (by vol.) of water, and its content determined spectrophotometrically (correction was made with respect to the blank performed in the absence of the EII protein fraction). The following buffer solutions were used in degradations: 0·05M sodium acetate for pH 4·0—6·0 and 0·05M Tris-HCl for pH 6·8—9·0 (by 0·3 pH). For the results see Fig. 1.

Effect of Some Ions on Degradation with the EII Protein Fraction

Degradation of uridine 2′,3′-cyclic phosphate (3 μmol of the lithium salt) in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·3) containing 0·1M-NaCl was performed in the presence the EII protein fraction (100 μg) and the appropriate inhibitor. After 1 h at 37°C, the incubation mixture was chromatographed in the solvent system S_1 and the spots processed as above. The RNA was degraded in 0·05M sodium acetate (pH 5·5) containing 0·1M-NaCl analogously to the pH-dependence assays. For inhibitors, concentration of inhibitors, and inhibition data see Table II.

Thermal Inactivation of the EII Protein Fraction in Some Degradations

To a solution of uridine 2′,3′-cyclic phosphate lithium salt (3 μmol) in 50 μl of 0·05M Tris-HCl buffer solution (pH 7·8) containing 0·1M-NaCl there was added a solution (preheated to 60°C

and rapidly cooled in an ice bath) of the EII protein fraction (63 μg) in the same buffer (50 μl). After 1 h at 37°C, the incubation mixture was chromatographed in the solvent system S_1 and the spots processed as above. The RNA was degraded in 0·05M sodium acetate (pH 5·5) containing 0·1M-NaCl analogously to the above pH-dependence of RNA degradation. The EII protein fraction was inactivated at 60°C as above. For the spectrophotometrically determined values see Table I.

The authors are indebted to Czechoslovak Academy of Sciences and Professor Dr V. Herout, Director of this Institute, for making possible the scientific stage of one of them (M. P.) in the Institute.

REFERENCES

1. Holý A., Plšek M., Haragsim O.: This Journal *41*, 1598 (1976).
2. Holý A.: This Journal *39*, 310 (1974).
3. Ivanova G. S., Holý A., Zelinková E., Bezborodova S. I., Abrosimova-Amelyanchik N. M., Tatarskaya R. I.: This Journal *39*, 2986 (1974).
4. Ivanova G. S., Holý A., Bezborodova S. I.: This Journal *39*, 993 (1974).
5. Holý A., Grozdanovič J.: Biochim. Biophys. Acta *277*, 556 (1972).
6. Kaiser P., Bonacker L., Witzel H., Holý A.: Hoppe-Seyler's Z.Physiol. Chem. *356*, 143 (1975).
7. Egami F., Nakamura K.: *Microbial Ribonucleases*. Springer Verlag, New York 1969.
8. Nikai M., Minami Z., Yamazaki T., Tsugita A.: J. Biochem. (Tokyo) *57*, 96 (1965).
9. Tang W. T., Maretzki A.: Biochim. Biophys. Acta *212*, 300 (1970).
10. Torti G., Mapelli S., Soave C.: Biochim. Biophys. Acta *324*, 254 (1973).
11. Hara A., Yoshikara K., Watanabe T.: Nippon Nogei Kagaku Kaishi *44*, 3852 (1970); Chem. Abstr. *74*, 38545 (1971).
12. Laskowski M., sr in the book: *The Enzymes* (P. D. Boyer, Ed.), Volume IV, p. 313. Academic Press, New York 1971.
13. Singer M. F., Tolbert G.: Biochemistry *4*, 1319 (1965).
14. Heppel L. A., Ortiz P. J., Ochoa S.: Science *123*, 415 (1956).
15. Vasilenko S. K., Babkina G. T.: Biokhimiya *30*, 705 (1965).
16. Morais R., Blackstein M., Lamirande de G.: Arch. Biochem. Biophys. *121*, 711 (1967).
17. Walters T. L., Loring H. S.: J. Biol. Chem. *241*, 2870 (1966).
18. Freeman K. B.: Canad. J. Biochem. *42*, 1099 (1964).
19. Drummond G. I., Yamamoto M. in the book: *The Enzymes* (P. D. Boyer, Ed.), Volume IV, p. 355. Academic Press, New York 1971.
20. Po-Chao Lin P., Varner J. E.: Biochim. Biophys. Acta *276*, 454 (1972).
21. Vandepeute J., Huffaker R. C., Alvarez R.: Plant Physiol. *52*, 278 (1973).
22. Anraku Y.: J. Biol. Chem. *239*, 3420 (1964).
23. Holý A., Bald R. W., Šorm F.: This Journal *37*, 592 (1972).
24. Holý A. in the book: *Mechanisms and Control Properties of Phosphotransferases* (J. Hoffman, E. Böhm, Eds), p. 555. Akademie-Verlag, Berlin 1972.
25. Zadražil S. in the book: *Synthetic Procedures in Nucleic Acid Chemistry* (W. W. Zorbach R. S. Tipson, Eds), Volume II, p. 547. Interscience, New York 1973.
26. Blank A., Dekker C. A., Biochemistry *11*, 3956 (1972).
27. Markham R., Smith J. G.: Biochem. J. *1952*, 552.
28. *Properties of Nucleic Acid Derivatives*. Calbiochem, Los Angeles 1964.

Translated by J. Pliml.