SUBSTRATE REQUIREMENTS OF SOME MICROBIAL RIBONUCLEASES NON-SPECIFIC TO NATURAL BASES

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The specificity of ribonucleases from *Penicillium brevicompactum*, *P. claviforme*, and *P. chrysogenum*, and two *Aspergillus* enzymes (*A. clavatus* and ribonuclease T2 from *A. oryzae*) towards ribonucleoside 2′-3′-cyclic phosphates modified on the heterocyclic and sugar moiety, and towards diribonucleoside phosphate analogues has been investigated. *1)* All these enzymes can be classified as ribonucleate D-nucleotido-2′-transferases (cyclizing) (EC 2.7.7.17) that require an interaction of the heterocyclic base with the enzyme, in addition to the phosphate group binding. *2)* The (3′→5′)-character of the internucleotidic linkage and the presence of a 2′-hydroxylic function in the *ribo* configuration of the 3′-nucleotide is required for the substrate molecule. *3)* No specific group or grouping is required for the heterocyclic moiety of the substrate molecule. *4)* In the pyrimidine series, the enzyme affinity is not inhibited by substitution of the base at positions $N_{(3)}$, $C_{(4)}$ or $C_{(5)}$ with groups of an inductive, mesomeric or steric effect or bearing an electronegative charge. *5)* The differences among the particular enzymes with respect to the nucleoside conformation and their different affinity towards 1-(2,3-dihydroxypropyl)thymine 2′,3′-cyclic phosphate demonstrate that the similar enzymes from the different species or strains of microorganisms specifically differ in their substrate requirements.

Ribonucleases T1 and T2 from the takadiastase preparation (*Aspergillus oryzae*), the former being guanine-specific and the latter base-nonspecific, represent in combination with ribonuclease A of bovine pancreas the most important tools in the analysis of ribonucleic acids and their derivatives. The occurrence of similar ribonucleases in other fungal species was anticipated and later on confirmed in the case of *Aspergillus*, *Penicillium*, *Actinomyces*, *Neurospora*, *Ustilago*, *Rhizopus* and other microbial species (for a review see ref.[1-3]). The enzyme groups of *Neurospora* and *Ustilago* have been investigated in detail; because of their importance, the ribonucleases N1 and U2 are now produced commercially.

However, the ribonucleases T1 and T2 have been investigated only with respect to their requirements on the substrate molecules. In investigations on the similarity

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of ribonucleases isolated from different species or strains, it does not suffice to limit the scope of the work to examinations of the enzyme response towards the four "natural" ribonucleotide derivatives; only by a detailed substrate specificity study information may be obtained on differences in the substrate requirements within the enzyme group investigated. By means of the corresponding data it would be then possible to elucidate the character of the active and regulatory site and of processes which are involved in the enzyme-substrate interaction.

Recently, some ribonucleases resembling to a certain extent the ribonucleases T2 and U2 have been isolated in our Laboratory from various fungal culture media. It was of interest to investigate whether the substrate requirements of our enzymes are identical with those of ribonucleases T2 and U2. Undoubtedly, some kinetic deviations may be expected. It is however the qualitative response of the enzyme towards various structural effects which represents the central point of our present investigation.

The present paper deals with ribonucleases from *Penicillium brevicompactum*[4], *P. claviforme*[5], *P. chrysogenum*[6], and *Aspergillus clavatus*[7] all of which have been found "nonspecific" liberating the four major 3'-nucleotides on yeast RNA hydrolysis. The present observations are compared with data on ribonuclease T2 reported earlier in our[8–10] and other papers (*cf.*[1,2,11]) or completed where necessary. The choice of compounds under study was similar to that in our earlier investigations on the general substrate substitution effects (for a summary see ref.[12]).

The ribonuclease character of the above enzymes was concluded from the intermediary 2',3'-cyclic nucleotide formation upon RNA or polyribonucleotide

TABLE I
Characteristics of Fungal Non-Specific Ribonucleases

Property	RNase T2[1]	*A. clavatus*[17]	*P. brevicompactum*[18]	*P. chrysogenum*[6]	*P. claviforme*[19]
Molecular weight	36 200	27 000	34 300	35 000	37 000
Absorption max.	281	282	281	282	283
Absorption min.	252	251–252	251–252	251–252	252
OD_{max}/OD_{min}	2·5	2·0	2·6	2·3	2·7
Isoelectric point	5·0	4·0	4·0–5·0	4·0–5·0	4·5
pH Optimum for RNA digestion	4·5	5·8–6·0	5·2	4·5	6·5
pH Optimum for uridine 2',3'-cyclic phosphate hydrolysis	6·0–6·3	4·5–4·8	4·7	6·5	4·5

TABLE II

Splitting of Ribonucleoside 2',3'-Cyclic Phosphate and Their Analogues by RNase-T_2, *A. clavatus* (A), *P. brevicompactum* (B), *P. chrysogenum* (C), *P. claviforme* (D)

Abbreviations: + good substrate; ± traces of splitting after 24 h; numbers represent percentage of splitting.

No	Nucleoside Residue	RNase T_2	A	B	C	D
I	uridine	+	100	+	100	100
II	cytidine	+	100	+	67	63
III	5-methyluridine	+	100	+	100	100
IV	5-chlorouridine	+	100	+	53	53
V	5-bromouridine	+	66	+	66	65
VI	5-aminouridine	+	47	+	47	48
VII	5-dimethylaminouridine	+	47	+	49	48
VIII	5-diethylaminomethyluridine	49	44	57	47	44
IX	5-ethoxycarbonyluridine	+	24	+	25	20
X	5-carboxyuridine	+	+	+	100	+
XI	6-carboxyuridine	+	28	+	46	69
XII	6-methyluridine	+	0	0	0	0
XIII	5,6-dimethyluridine	+	0	0	0	0
XIV	5-methylcytidine	+	100	+	100	100
XV	N^4-dimethylcytidine	+	0	57	0	0
XVI	N^3-methyluridine	+	0	+	13	37
XVII	N^3-(2-hydroxyethyl)uridine	+	0	0	0	0
XVIII	isocytidine	+	0	0	0	0
XIX	2-pyrimidone 1-riboside	100	100	100	100	100
XX	pyrimidone 3-riboside	+	0	0	0	0
XXI	4-methyl-2-pyrimidone 1-riboside	100	100	199	100	100
XXII	6-methyluracil 3-riboside	+	0	0	0	0
XXIII	(*R*)-1-(2',3'-dihydroxypropyl)thymine[a]	0	+	+	+	+
XXIV	uric acid 3-riboside	+	18	100	100	5
XXV	6-azauridine	+	22	±	57	22
XXVI	5-methyl-6-azauridine	+	±	+	24	±
XXVII	N^3-methyl-6-azauridine	+	0	+	13	37
XXVIII	L-uridine	0	0	0	0	0
XXIX	α-uridine	0	0	0	0	0
XXX	thymine 1-β-D-ribopyranoside	0	0	0	0	0
XXXI	adenosine	100	100	100	100	100
XXXII	guanosine	100	100	100	100	100
XXXIII	inosine	+	40	+	57	20
XXXIV	xanthosine	+	0	±	0	0
XXXV	tubercidine	+	38	+	45	45
XXXVI	isoguanosine	+	0	0	0	0
XXXVII	purine 9-riboside	100	100	100	100	100

TABLE II
(*Continued*)

No	Nucleoside Residue	RNase T_2	A	B	C	D
XXXVIII	2-aminopurine riboside	+	±			
XXIX	2,6-diaminopurine riboside	+	±			
XL	6-thioinosine	+	18	+	100	40
XLI	N^6-dimethyladenosine	+	32	+	54	20
XLII	N^2-methylguanosine	7	0	30	7	0
XLIII	bromoguanosine	±	0	0	0	0
XLIV	8-aminoguanosine	±	13	6	0	0
XLV	8-hydroxyguanosine	+	0	+	0	0
XLVI	8-mercaptoguanosine	0	0	0	0	0
XLVII	L-adenosine	0	0	0	0	0
XLVIII	L-guanosine	0	0	0	0	0
XLIX	adenine α-L-lyxofuranoside	+	15	+	49	37
L	adenine α-D-lyxofuranoside	0	0	0	0	0
LI	hypoxanthine α-L-lyxofuranoside	+	67	+	79	82

[a] For preparation and detailed discussion, *cf.*[15].

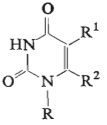
hydrolysis and by the cleavage of the ribonucleotide 2',3'-cyclic phosphates themselves to the corresponding 3'-ribonucleotides. The main features of the enzymes are summarized in Table I. The substrate specificity investigation was effected mainly with nucleoside 2',3'-cyclic phosphates modified on the base and sugar moiety (Table II). Consequently, the thus-obtained information relates theoretically to the hydrolytic step of the ribonucleate hydrolysis and not strictly to the phosphate activation (transfer) reaction. However, these findings may be used in investigations on the enzyme recognition site. Furthermore, some dinucleoside phosphate analogues and other compounds have been examined; the corresponding data are summarized in Table III.

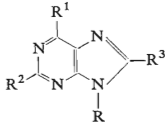
The data obtained in investigations on the above substrates and substrate analogues can be interpreted in conclusions of a general validity for the whole group of enzymes and in terms of the special structural requirements of the particular enzymes.

General Features and Substrate Structural Requirements

The *Penicillium* and *Aspergillus* ribonucleases cleave ribonucleic acid and polyribonucleotides to ribonucleoside 3'-phosphates *via* an intermediary formation of ribonucleoside 2',3'-cyclic phosphates. There is no specificity with respect to the four "natural" bases in ribonucleic acids. The ribonucleoside 2',3'-cyclic phosphates deri-

ved from adenosine, guanosine, uridine, and cytidine are also cleaved to the corresponding 3′-nucleotides. The enzymes cleave specifically the (3′ → 5′)-linkages, leaving the (2′ → 5′)- and (5′ → 5′)-linkages intact (*cf.* compounds *LII* – *LIV*). The cleavage of the (3′ → 5′)- internucleotidic linkage requires the presence of a vicinal 2′-hydroxylic function in the *ribo* configuration on the molecule of the 3′-nucleotide residue. If this group is absent (compound *LV*), etherified by the tetrahydropyranyl residue (*LIX*) or a methyl group (*LVII*), replaced by the chlorine atom (*LVIII*) or converted to the *arabino* configuration (*LVI*), the substrate activity completely disappears. Consequently, the intermediary 2′,3′-cyclic phosphodiester formation is essential for the cleavage of the (3′ → 5′)-internucleotidic linkage.





I; $R^1 = R^2 = H$

III; $R^1 = CH_3$, $R^2 = H$

IV; $R^1 = Cl$, $R^2 = H$

V; $R^1 = Br$, $R^2 = H$

VI; $R^1 = NH_2$, $R^2 = H$

VII; $R^1 = N(CH_3)_2$, $R^2 = H$

VIII; $R^1 = (C_2H_5)_2NCH_2$, $R^2 = H$

IX; $R^1 = COOC_2H_5$, $R^2 = H$

X; $R^1 = COOH$, $R^2 = H$

XI; $R^1 = H$, $R^2 = COOH$

XII; $R^1 = H$, $R^2 = CH_3$

XIII; $R^1 = R^2 = CH_3$

XXIV; $R^1 = R^2 =$ —NHCONH—

XXVIII; $R^1 = R^2 = H$, R = β-L-ribofuranoside 2′,3′-cyclic phosphate

XXIX; $R^1 = R^2 = H$; R = α-D-ribofuranoside 2′,3′-cyclic phosphate

XXX; $R^1 = CH_3$, $R^2 = H$, R = β-D-ribopyranoside 2′,3′-cyclic phosphate

XXXI; $R^1 = NH_2$, $R^2 = R^3 = H$

XXXII; $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$

XXXIII; $R^1 = OH$, $R^2 = R^3 = H$

XXXIV; $R^1 = R^2 = OH$, $R^3 = H$

XXXVI; $R^1 = NH_2$, $R^2 = OH$, $R^3 = H$

XXXVII; $R^1 = R^2 = R^3 = H$

XXXVIII; $R^2 = NH_2$, $R^1 = R^3 = H$

XXXIX; $R^1 = R^2 = NH_2$, $R^3 = H$

XL; $R^1 = SH$, $R^2 = R^3 = H$

XLI; $R^1 = N(CH_3)_2$, $R^2 = R^3 = H$

XLII; $R^1 = OH$, $R^2 = NHCH_3$, $R^3 = H$

XLIII; $R^1 = OH$, $R^2 = NH_2$, $R^3 = Br$

XLIV; $R^1 = OH$, $R^2 = R^3 = NH_2$

XLV; $R^1 = R^3 = OH$, $R^2 = NH_2$

XLVI; $R^1 = OH$, $R^2 = NH_2$, $R^3 = SH$

XLVII; $R^1 = NH_2$, $R^2 = R^3 = H$, R = = β-L-ribofuranoside 2′,3′-cyclic phosphate

XLVIII; $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$, R = β-L-ribofuranoside 2,3-cyclic phosphate

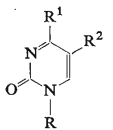
XLIX; $R^1 = NH_2$, $R^2 = R^3 = H$, R = = α-L-lyxofuranoside 2′,3′-cyclic phosphate

L; $R^1 = NH_2$, $R^2 = R^3 = H$, R = = α-D-lyxofuranoside 2′,3′-cyclic phosphate

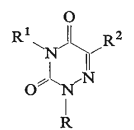
TABLE III

Splitting of Other Nucleotidic Derivatives by RNase-T_2, *A. clavatus* (A), *P. brevicompactum* (B), *P. chrysogenum* (C), *P. claviforme* (D)

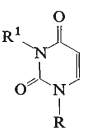
No	Compound	RNase T_2	A	B	C	D
LII	(2′ → 5′)-UpU	0	0	0	0	0
LIII	(2′ → 5′)-UpA	0	0	0	0	0
LIV	(5′ → 5′)-UpU	0	0	0	0	0
LV	dUpdC	0	0	0	0	0
LVI	araCpU	0	0	0	0	0
LVII	2′-O-methyluridylyl-(3′ → 5′)-uridine	0	0	0	0	0
LVIII	2′-chloro-2′-deoxyuridylyl-(3′ → 5′)-uridine	0	0	0	0	0
LIX	2′-O-tetrahydropyranylguanylyl-(3′ → 5′)-uridine	0	0	0	0	0
LX	adenosine 3′,5′-cyclic phosphate	0	0	0	0	0
LXI	methyl 3′-cytidylate	+	84	+	95	37
LXII	methyl 2′-cytidylate	0	0	0	0	0
LXIII	ethyl 3′adenylate	+	37	57	69	74
LXIV	1-propyl 3′-adenylate	+	27	+	69	18
LXV	1-butyl 3′adenylate	+	23	+	68	15
LXVI	2-propyl 3′-adenylate	+	10	65	42	83



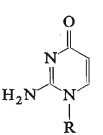
II; $R^1 = NH_2$, $R^2 = H$
XIV; $R^1 = NH_2$, $R^2 = CH_3$
XV; $R^1 = (CH_3)_2N$, $R^2 = H$
XIX; $R^1 = R^2 = H$
XXI; $R^1 = CH_3$, $R^2 = H$



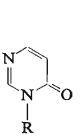
XXV; $R^1 = R^2 = H$
XXVI; $R^1 = H$, $R^2 = CH_3$
XXVII; $R^1 = CH_3$, $R^2 = H$



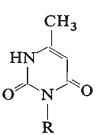
XVI; $R^1 = CH_3$
XVII; $R^1 = HOCH_2CH_2$



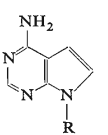
XVIII



XX



XXII

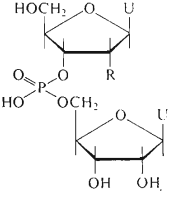


XXXV

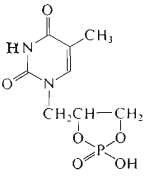
If not stated otherwise, in all formulae R = β-D-ribofuranoside 2′,3′-cyclic phosphate

The enzymes split specifically the 2′,3′-cyclic phosphates of the D-series only, while the L-enantiomers (*XXVIII*, *XLVII*, *XLVIII*) remain intact. It may be inferred from these observations[12] that the substrate molecule interacts with the enzyme molecule at least at three different sites including the chiral portion (the nucleoside moiety) of the substrate molecule. All the ribonucleases examined show this feature[12]; as it may be assumed that two of the above fixation points correspond to the phosphorus atom binding or to the interaction at the active site, the third point remains for the interaction of the heterocyclic base with the polypeptide chain of the enzyme at the regulatory site. The enzymes examined thus evidently require the presence of a heterocyclic base in the particular conformation; only the β-D-ribonucleotides correspond to this requirement in contrast to the L-enantiomers *XXVIII*, *XLVII* and *XLVIII* or the α-anomers of the D-*ribo* series (*XXIX*). Furthermore, as it may be inferred from the nonspecificity for the pyrimidine or purine nucleotides of the "natural" series as well as from data on various modified nucleotides, the forces involved in this interaction of heterocyclic bases do not depend on the presence of any specific group. Hence, the base cannot be bound or "recognised" by the enzyme molecule as in the case of specific ribonucleases (ribonuclease A, ribonuclease T1). The character of the interaction might possess the features of a $\pi-\pi$ interaction with the heteroaromatic ring system of the base. Conclusively, the enzymes examined may be classified as D-ribonucleate nucleotido-2′-transferases (cyclizing) (EC 2.7.7.17), similarly to ribonuclease T2.

In the series of nucleotide alkyl esters, both ribonuclease T2 and the group of enzymes examined are capable of splitting the 3′-ribonucleotide alkyl esters without any special sensitivity concerning the character, chain-length or steric requirements of the alkyl group (*cf.* compounds *LXI*, *LXIII*–*LXVI*). In this respect, these enzymes



U = uracil
LVII; R = OCH_3
LVIII; R = Cl
LIX; R = O–



(*RS*)-*XXIII*

differ from the spleen cyclic phosphodiesterase which is dependent upon the effects mentioned[13] and which, similarly to pancreatic ribonuclease, is not capable of splitting the 2-propyl esters at all. As expected, the 2'-nucleotide alkyl ester *LXII* is quite resistant to the action of all the enzymes mentioned. Consequently, there is no special role of the nucleoside bound as an ester group to the 3'-nucleotide in an internucleotidic linkage. Its presence, however, may exhibit a cooperative effect upon the ES-complex stabilisation. This information could be obtained solely on the basis of kinetic measurements.

Within the group of pyrimidine ribonucleoside 2',3'-cyclic phosphates (Table II), there is no qualitative effect due to substitution at position 5 of the pyrimidine ring by an alkyl group, halogen atom, amino group, N,N-dialkylamino group, carboxyl or N,N-dialkylaminomethyl group (*cf.* compounds *I*, *II*, *III–X*, *XIV*). Neither of these substituents appears to display a steric or inductive effect. Furthermore, neither the introduction of 6-carboxy group (the orotidine derivative *XI*) does result in any loss of the substrate affinity. These properties are common for the whole group examined, including the RNase T2.

Similarly to ribonuclease T2, the enzymes examined split the N^3-methyl derivatives of uridine (*XVI*) and 6-azauridine (*XXVII*) nucleotides. However, the overall affinity of the 6-azauridine derivatives is lower than that of the uridine series (*cf. XXV–XXVII*).

In the purine nucleotide series, all the enzymes including ribonuclease T2 split adenosine, guanosine, inosine, and purine ribonucleoside nucleotides. Moreover, the 6-thioinosine derivative *XL* and the N^6-dimethyladenosine derivative *XLI* are readily split by all the enzymes. The other substituent effects are different with particular enzymes and are dealt with later.

The change of configuration of the 4'-hydroxymethyl group on the sugar moiety as accomplished with the α-L-lyxofuranosyl derivatives *XLIX* and *LI*, does not appreciably affect the substrate affinity when compared with the corresponding riboside derivatives *XXXI* and *XXXIII*. Consequently, the relative orientation of the heterocyclic base and the 4'-hydroxymethyl group does not appear important for the substrate affinity. As expected, the *D*-enantiomer *L* corresponding to the L-ribonucleotide *XLVII*, is quite resistant to all the enzymes. In contrast to the configuration at position 4', the change of the sugar ring from that in the ribofuranoside 2',3'-cyclic phosphate *III* to that in the ribopyranoside derivative *XXX* results in a complete loss of affinity towards all the enzymes examined. Thus, the mutual orientation of the heterocyclic base and the phosphate (or the 2',3'-cyclic phosphate) moiety is very important for the proper interaction of the substrate with the enzyme molecule. The change of such an orientation is more profound in the adenosine 3',5'-cyclic phosphate (*LX*) which is quite resistant towards the whole enzyme group of *Aspergillus* and *Penicillium*.

Specific Substrate Requirements of Aspergillus *and* Penicillium *Ribonucleases.*

The particular enzymes will be discussed with respect to the properties different from those of the other members of the above group.

In contrast to the other four enzymes, ribonuclease T2 (*Aspergillus oryzae*) is not sensitive to the substitution of the pyrimidine ring at position 6 by the methyl group (*cf.* compounds *XII* and *XIII*). Since this substitution probably results in a conformational change of the nucleoside moiety[10], the lack of affinity with the other ribonucleases appears consistent with the steric hindrance of the base–enzyme interaction at the regulatory site. The steric effect of the substituent at position 3 of the pyridine ring is also different throughout the enzyme group; while the N^3-methyl group does not bring about any remarkable effect (*vide supra*), the N^3-(2-hydroxyethyl) substituent (*cf.* compound *XVII*) causes lack of activity with all the enzymes except for ribonuclease T2. A similar effect has been observed with substituents at $C_{(4)}$ atom of the pyrimidine ring. While the introduction of a hydrogen atom (*XXIX*), a methyl group (*XXI*), a hydroxyl (*I*) or an amino group (*II*) into this position leads to active substrates for all the enzymes, the $C_{(4)}$ substitution by the bulky N,N-dimethylamino group (compound *XV*) results in loss of the substrate activity with all the enzymes except for ribonuclease T2. In conclusion, all the four enzymes except for ribonuclease T2 display an increased sensitivity towards substitution at positions 3 and 4 of the pyrimidine ring by bulky substituents. Since the methyl group of the 6-methyluridine derivatives, as a result of the conformational change, will approach position 2 of nucleosides in an *anti* conformation, the region sensitive to the bulky substitution may be extended in pyrimidine compounds to the whole surroundings of the base located away from the sugar-phosphate moieties.

Another point in which ribonuclease T2 markedly differs from the remaining four ribonucleases relates to the lack of sensitivity towards changes in the π-electron distribution caused by substitution at $C_{(6)}$ of the pyrimidine ring by an oxo group (compounds *XX* and *XXII*) or by replacement of the 2-oxo group by an amino group (the isocytidine derivative *XVIII*). Experiments with homonucleotides[14] have already shown that the enzyme region responsible of the interaction with the base moiety of the substrate molecule must be much larger than in the remaining enzymes examined. Consistent with this idea is also the observation that the 8-substituted guanosine derivatives (*XLIII*–*XLVI*) are poor substrates if any for the enzymes examined except for ribonuclease T2. In compounds *XLIII*–*XLVI*, the change of the nucleoside conformation from *syn* to *anti* (due to substituents) results in declination of the aromatic π-electron system (the pyrimidine part) of the purine heterocyclic system from the sugar-phosphate moiety. Consequently, the cooperative interaction with the heterocyclic base is lowered in all cases except for ribonuclease T2; the probability of an ES complex formation is thus decreased.

Noteworthy is the lack of activity of all the enzymes examined except for ribonuclease T2 to xanthosine 2′,3′-cyclic phosphate. This inactivity can be hardly due to the considerably high acidity of the heterocyclic base since the strongly acidic

pyrimidine derivatives *X*, *XI*, and *XXV* are split by all the enzymes discussed. A similar behaviour has been also encountered with the isoguanosine derivative *XXXVI* which is again enzyme-resistant with the exception of ribonuclease T2.

Finally, there is a very important difference between ribonuclease T2 and the remaining enzymes consisting in the negative response of ribonuclease T2 towards the aliphatic nucleotide analogue, namely, 1-(2′,3′-dihydroxypropyl)thymine 2′,3′-cyclic phosphate (*XXIII*). Compound *XXIII* is the single analogue resistant to ribonuclease T2 in contrast to the remaining enzymes examined. Presumably only a compound of the D-*glycero* configuration may imitate the conformation of a ribonucleoside derivative. As the requirements of all the enzymes examined except for ribonuclease T2 are obviously critical with respect to the sterical arrangement of the substrate molecule (*vide supra*), the reason of the failure with ribonuclease T2 might consist in the absence of some part of the sugar moiety groupings in the analogue molecule. The influence of the presence of the riboside 4′-hydroxymethyl group may be excluded as it has been shown that the sole of this group is negligible. On the other hand, it appears very plausible that ribonuclease T2 requires the presence of a cyclic acetal oxygen atom at position 6′. In this respect, ribonuclease T2 is similar to the pancreatic ribonuclease A, snake venom phosphodiesterase, spleen acid exonuclease or seminal ribonuclease which are also unable to cleave compound *XXIII* (*cf.*[15]).

The structural requirements of the *P. brevicompactum* ribonuclease have been described above. There are only a few exceptions in which this particular enzyme differs from the other two *Penicillium* ribonucleases, such as the low sensitivity to the bulky substituents at positions 3 and 4 of the pyrimidine ring (compounds *XV* and *XVI*); in this respect, there is a greater resemblance to ribonuclease T2. The most striking observation made with the title enzyme relates to the fair activity towards the N^2-methylguanosine derivative *XLII* which is otherwise a very poor substrate for the remaining enzymes examined including ribonuclease T2.

The *P. claviforme* and *P. chrysogenum* ribonucleases differ substantially from ribonuclease T2 and the *P. brevicompactum* ribonuclease in points mentioned above. From the other two *Penicillium* ribonucleases, the *P. claviforme* enzyme differs merely by the negligible affinity to compound *XXIV i.e.*, uric acid 3-riboside derivative.

The *A. clavatus* ribonuclease behaves similarly to the *Penicillium* enzymes in respects mentioned above and is entirely different from ribonuclease T2, the other *Aspergillus* enzyme. The only point in which this enzyme differs from all the remaining enzymes examined consists in the small affinity towards the 6-azauridine derivatives *XXV*–*XXVII*.

From the above data on the five so called nonspecific ribonucleases of fungal origin (two *Aspergillus* and three *Penicilium* enzymes) it may be concluded that the enzymes behave in a quite different way towards substrates modified by substituents leading to steric effects, conformational changes, changes in π-electron distribu-

tion, and other effects. Thus, the situation at the regulatory sites of the enzymes must be quite different. The above results show how dangerous would it be to regard the apparently similar enzymes even of the different strains of the species as identical in their substrate requirements or with respect to the situation at the regulatory site. Numerous modifications of the substrate are necessary to determine the response of the particular enzyme to changes due to various substituents. Similar differences in the behaviour of enzymes might be detected with the use of kinetic techniques at the active sites. The above findings suggest the necessity to collect additional data on differences in properties of apparently similar or related ribonucleases.

EXPERIMENTAL

Extracellular ribonucleases from *Penicillium brevicompactum*[4], *P. chrysogenum*[6], *P. claviforme* and the intracellular ribonuclease of *Aspergillus clavatus*[7] were prepared as described earlier. Ribonuclease T2 of *Aspergillus oryzae* was kindly provided by Professor H. Witzel, University Münster, German Federal Republic. The preparation of substrates has been reported earlier[13]. The estimation of the enzymatic activity of the above ribonucleases was effected by measurements of the acid-soluble products obtained by digestion of yeast RNA according to a modified method as described earlier[16]. Since the pH optimum of ribonucleoside 2′,3′-cyclic phosphates and the short-chain oligonucleotides lies (similarly to the RNase T2) at pH 5—6 (Table I), the experiments were performed in sodium β,β-dimethylglutarate buffer solution (pH 6·0) to lower the non-enzymatic hydrolysis. Such an enzyme concentration was used as sufficient to hydrolyze just completely uridine 2′,3′-cyclic phosphate and adenosine 2′,3′-cyclic phosphate in the course of 3 h.

Assay of hydrolase activity. The reaction mixture contained 2 μmol of the test substance in 60 μl of 0·05M sodium β,β-dimethylglutarate (pH 6·0) and the corresponding enzyme, *i.e.*, 0·009 unit *A. clavatus* RNase, 0·04 unit *P. chrysogenum* RNase, 0·1 unit *P. claviforme* RNase, 35 units *P. brevicompactum* RNase, and 20 μg RNase T2. After 4 and 24 h at 37°C aliquots of the mixtures were withdrawn and applied to Whatman No 3 MM paper. Blanks were performed analogously but in the absence of enzymes. Spots of the starting material and products obtained by descending chromatography (24 h) in 2-propanol–conc. NH_4OH–water (7 : 1 : 2) were eluted with water (10 ml) and the content of the eluate was estimated spectrophotometrically at 260 nm on a Beckman DU apparatus. The values obtained were corrected for the non-enzymatic hydrolysis (determined by blanks) and summarized in Tables II and III (the 24 h values only are tabulated). Values exceeding by 10% the non-enzymatic hydrolysis were considered significant.

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