SUBSTRATE SPECIFICITY OF SOME MICROBIAL GUANYLORIBONUCLEASES

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Five guanyloribonucleases, namely, ribonuclease T 1 of *Aspergillus oryzae*, the extracellular ribonuclease C 2 of *Aspergillus clavatus*, the intracellular ribonuclease II of *Aspergillus clavatus*, and the ribonucleases from *Actinomyces aureoverticillatus* and *Streptomyces aureofaciens* were investigated with respect to their affinity towards ribonucleoside 2',3'-cyclic phosphates with modified heterocyclic bases, analogues with modified sugar moieties, and towards diribonucleoside phosphates derived from naturally occurring nucleosides. All the five enzymes are very similar to each other in their requirements on substrate molecules; they belong to D-ribonucleotide 2'-transferases (cyclising) (EC 2.7.7.26) specific for 3'-guanylic or 3'-inosinic acid esters of the natural series. In general, the requirement for a strictly localised —NH—CO— group has to be fulfilled by the substrate molecule. The binding of the heterocyclic base is necessary for the formation of the enzyme-substrate complexes. The presence of a nitrogen atom (probably its lone electron pair) at position 7 of the purine ring is necessary for the binding. Ribonuclease TI differs from the other enzymes by the lack of affinity towards the 6-thioinosine derivative. In the diribonucleoside phosphate series, 3'-guanylic acid esters are split preferentially; application of a greater excess of the enzyme leads to a considerable non-specific transfer reaction yielding the ribonucleoside 2',3'-cyclic phosphate and the ribonucleoside.

Since the discovery of ribonuclease T 1 from the Takadiastase preparation of *Aspergillus oryzae* which became a perfect tool for sequential studies of ribonucleic acids[1], the systematic search for similar ribonucleases specific for guanylic acid diesters resulted in isolation of some other enzymes from fungal species. The common features of all these ribonucleases consist in a low molecular weight (10–15000),

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thermal stability, and specific cleavage of 3'-guanylic (or 3'-inosinic) acid diesters *via* the corresponding 2',3'-cyclic phosphates. Ribonuclease U 1 of *Ustilago sphaerogena* and ribonucleases N 1 and N 3 of *Neurospora crassa* belong to the best known enzymes of this type[2]. In our Laboratories, a similar guanyloribonuclease has been isolated from *Actinomyces aureoverticillatus*[3] and found to possess properties very similar to those of ribonuclease T 1, both in the cleavage reaction[4] and the synthetic reaction[5]. Recently, two low-molecular weight ribonucleases have been isolated from *Aspergillus clavatus*, probably also guanyloribonucleases; one of them was eliminated into the cultural medium while the other one is located inside the cells[6,7]. An extracellular enzyme has been recently discovered in the chlorotetracycline-producing strain of *Streptomyces aureofaciens*[8].

Substantial differences in the substrate specificity of "non-specific" ribonucleases isolated from similar microorganisms or from different species of the same microorganism shown in an earlier paper[9] prompted us to investigate the analogous problem in the series of base-specific ribonucleases. Previously[10], we have demonstrated a practically identical substrate specificity of two pyrimidine-specific ribonucleases from the bovine pancreas (ribonuclease A) and bovine seminal plasma (ribonuclease AS, ribonuclease S 1, *cf.*[11,12]) representing two different low-molecular weight proteins from different tissues of the same animal. It was also of interest to find out whether the strict substrate specificity might be a general property of the base-specific low-molecular enzymes.

For this purpose, the affinity of five guanyloribonucleases has been compared, namely, of the T 1 enzyme of *Aspergillus oryzae*, the extracellular ribonuclease (ribonuclease C 2) of *Aspergillus clavatus*, the intracellular ribonuclease (ribonuclease II) of the same strain, the *Actinomyces* ribonuclease of *Actinomyces aureoverticillatus*, and the ribonuclease of *Streptomyces aureofaciens*. In this set, there are enzymes from two different species of *Aspergillus*, two enzymes of the same microorganism differing in their localisation, and finally, enzymes from two different classes of microorganisms. The main features of these enzymes such as their ribonuclease character and the specificity towards the guanylic acid residue, are known from earlier papers[4,6-8]. In the present paper, the specificity of the above mentioned enzymes has been examined in detail on nucleoside 2',3'-cyclic phosphates modified on the heterocyclic or sugar moiety and on diribonucleoside phosphates. The enzyme concentration was maintained high enough to ensure the hydrolytic reaction (if any). As a result of this study, there was obtained an information on the requirements of the regulative sites of the present enzymes while the elucidation of the active site similarity would be accessible by kinetic measurements and physical investigations only.

The general characteristics of the present enzymes are shown in Table I. The data indicate a great similarity of these proteins in many aspects such as the molecular weight, spectroscopic parameters, and optimum pH values for the RNA hydrolysis.

Unfortunately, there are only few data available on the hydrolysis of low-molecular weight substrates by means of the present enzymes. The investigations of Irie[13] and Pongs[14] on ribonuclease T 1 suggest the complex character of the pH dependence of K_M and k_{cat} (or v_{max}) values with dinucleoside phosphates and guanosine 2′,3′-cyclic phosphate. It appeared therefore advisable to standardize in the present work the enzyme concentration by a RNA digestion test and furthermore, to perform all the experiments at pH 6. In some special cases, the cleavage was also examined in the more acidic pH region. To get a reliable information, the incubation mixtures were analysed after a short and a prolonged incubation period of time and the cleavage was determined with the use of a blank performed under analogous conditions to eliminate the non-enzymatic hydrolysis, the extent of which may be considerably high in some cases.

Our data support the earlier findings[2,3,7,15,16] on the ribonuclease character of the present enzymes. In contrast to GpU which is split by all the present enzymes to guanosine 3′-phosphate and uridine (with the intermediary formation of guanosine 2′,3′-cyclic phosphate), the protected analogue, namely, 2′-O-tetrahydropyranyl-guanylyl-(3′ → 5′)-uridine, is completely resistant towards all the present enzymes. Thus, the 2′-hydroxylic function is undoubtedly necessary to accomplish the first step, *i.e.*, the transfer reaction.

TABLE I

Properties of Guanyloribonucleases[a]

Property	A	B	C	D	E
Molecular weight	11 000	11 150	13 000	13 000	9 000
Absorption maximum	278	278	278	278	
Absorption minimum	251—252	250—251	252—253	253—254	
OD_{max}/OD_{min}	3·0	3·14	2·7	1·6	
$OD_{max}^{0.1\%}$	1·9	1·9	1·68		
Isoelectric point	2·9	~4·0	4·3	4·0	4·3
pH Optimum for RNA hydrolysis	7·4	8·0	8·0	7·6—7·8	7·0
pH Optimum for guanosine 2′,3′-cyclic phosphate hydrolysis	7·0		4·5—5·0		5·7

[a] A ribonuclease T_1 (*Asp. oryzae*)[2]; B ribonuclease C_2 (*Asp. clavatus*)[6]; C, ribonuclease II (*Asp. clavatus*)[28]; D, *Act. aureovert.* ribonuclease[4]; E, *Str. aureofaciens* ribonuclease[16].

Table II shows the data obtained in examination of ribonucleoside 2′,3′-cyclic phosphates containing modified heterocyclic bases and of analogues modified in the sugar moiety. The striking similarity in behaviour of the five enzymes towards monomeric substrates may be expressed as follows.

1. All the present enzymes split preferentially guanosine 2′,3′-cyclic phosphate and inosine 2′,3′-cyclic phosphate. Concerning xanthosine 2′,3′-cyclic phosphate, best results were obtained with the *Streptomyces* ribonuclease, the remaining four enzymes being rather poor in this respect. Neither uridine derivatives nor cytidine derivatives are cleaved by any of the five enzymes. This behaviour is similar to that

TABLE II

Substrate Specificity of Guanyloribonucleases[a] as Determined by Splitting of Nucleoside 2′,3′-Cyclic Phosphates

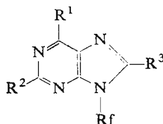
No	Nucleoside residue	Splitting, %				
		A	B	C	D	E
I	guanosine	100	67	83	44	70
II	inosine	100	100	100	42	50
III	xanthosine	+	9	10	11	70
IV	L-guanosine	0	0	0	0	0
V	L-adenosine	0	0	0	0	0
VI	adenosine	0	0(3)	20[b]		0
VII	uridine	0	0	0	0	0
VIII	cytidine	0	0	0	0	0
IX	isocytidine	5	0	0	0	27
X	2-aminopurine riboside	0	6	6	0	0
XI	2,6-diaminopurine riboside	0	5	6	0	0
XII	isoguanosine	0	0	0	0	0
XIII	purine riboside	0	0	0	0	±
XIV	tubercidine	0	0	0	5	0
XV	N^2-methylguanosine	+	35	42	21	60
XVI	6-thioguanosine	0	6	9	10	100
XVII	8-bromoguanosine	100	48	62	16	58
XVIII	8-aminoguanosine	100	51	100	56	+
XIX	8-hydroxyguanosine	±[c]	5	5	8	+
XX	8-mercaptoguanosine	±[c]	0	0	0	0
XXI	hypoxanthine α-L-lyxofuranoside	+	30	34	[d]	[d]
XXII	adenine α-L-lyxofuranoside	0	0	0	0	0
XXIII	adenine α-D-lyxofuranoside	0	0	0	0	[d]
XXIV	uric acid 3-riboside	0	0	0	0	20
XXV	deazainosine	0	[d]	[d]	[d]	0

[a] For symbols A—E see Table I; [b] 26% splitting at pH 4·5; [c] trace of splitting; [d] not known.

of ribonuclease T 1 (*cf.*[2]). From the five enzymes, only ribonuclease II of *Aspergillus clavatus* exhibits an affinity towards adenosine 2′,3′-cyclic phosphate similar to that of ribonuclease U 2, the purine-specific enzyme[17].

2. The lack of affinity towards the L-guanosine derivative *IV* was shown by all the present enzymes. It may be assumed analogously to ribonuclease T 1 (*cf.*[18]) and some other ribonucleases[19] that the formation of the enzyme-substrate complex requires fixation of the nucleotide in three different points, two of them being mediated by the phosphate group while in the remaining third point there is involved the chiral nucleoside moiety which must fulfil the requirements of the enzyme in the interaction with the heterocyclic base. The recognition site should be large enough to allow acceptance of the enantiomer (*cf.*[20]).

3. As shown by investigations on 2,6-disubstituted purine derivatives, all the five enzymes require the presence of the —NH—CO— grouping at positions 1 and 6 of the pyrimidine portion of the purine ring system. Thus, neither the purine derivative *XIII* itself, nor its 2-amino(*X*) and 2,6-diamino (*XI*) derivative and the isoguanosine derivative *XII* are substrates for the enzymes. Such a behaviour is again consistent with that of ribonuclease T 1. Replacement of the 6-oxo group by the 6-thio function in the 6-thioguanosine derivative *XVI* results in a loss of activity in the case of ribonuclease T 1 (*cf.*[21–23]); on the other hand, a slight but distinct cleavage reaction takes place with the present enzymes after a prolonged reaction period of time. This reaction is quite remarkable with the *Streptomyces* ribonuclease which splits the



I, $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$
II, $R^1 = OH$, $R^2 = R^3 = H$
III, $R^1 = R^2 = OH$, $R^3 = H$
IV, $R^1 = OH$, $R^2 = NH_2$, $R^3 = H$
Rf = β-L-ribofuranoside 2′,3′-cyclic phosphate
V, $R^1 = NH_2$, $R^2 = R^3 = H$,
Rf = β-L-ribofuranoside 2′,3′-cyclic phosphate
VI, $R^1 = NH_2$, $R^2 = R^3 = H$
X, $R^2 = NH_2$, $R^1 = R^3 = H$
XI, $R^1 = R^2 = NH_2$, $R^3 = H$
XII, $R^1 = R^2 = NH_2$, $R^3 = H$
XIII, $R^1 = R^2 = H$

XV, $R^1 = OH$, $R^2 = CH_3NH$, $R^3 = H$
XVI, $R^1 = SH$, $R^2 = NH_2$, $R^3 = H$
XVII, $R^1 = OH$, $R = NH_2$, $R^2 = Br$
XVIII, $R^1 = OH$, $R^2 = R^3 = NH_2$
XIX, $R^1 = R^3 = OH$, $R^2 = NH$
XX, $R^1 = OH$, $R^2 = {}_2$,NH $R^3 = SH$
XXI, $R^1 = OH$, $R^2 = R^3 = H$
Rf = α-L-lyxofuranoside 2′,3′-cyclic phosphate
XXII, $R^1 = NH_2$, $R^2 = R^3 = H$,
Rf = α-L-lyxofuranoside 2′,3′-cyclic phosphate
XXIII, $R^1 = NH_2$, $R^2 = R^3 = H$,
Rf = α-D-lyxofuranoside 2′,3′-cyclic phosphate

6-thioguanosine derivative *XVI* much better than the guanosine derivative *I*. This difference might be ascribed to lower requirements on the spatial relations of the 6-substituent or its polarisability when compared with ribonuclease T 1.

4. The presence of the —NH—CO— grouping in the pyrimidine portion only is insufficient for recognition of the substrate by the enzyme. This grouping is also present in the uridine derivative *VII*, the isocytidine derivative *IX* or the uric acid 3-riboside derivative *XXIV* but nevertheless, none of these compounds is split by ribonuclease T 1 or the present *Aspergillus* or *Actinomyces* enzymes. Consequently, the correct mutual orientation of the binding and recognition sites is necessary for the enzyme-substrate complex formation. As pointed out earlier[14], the difference between the pyrimidine and purine derivatives carrying the same —NH—CO— grouping is about 2 Å; this observation supports the idea on the strict conformational requirements of the enzymes. It is of interest to mention the exceptional behaviour of the *Streptomyces aureofaciens* enzyme which splits compounds *IX* and *XXIV*. In this case, the enzyme-substrate complex can be thus formed even under such conditions which would be insufficient with other members of the group under investigation.

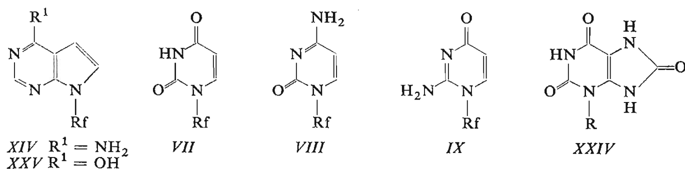
TABLE III

Splitting of Diribonucleoside Monophosphates with *Aspergillus* Guanyloribonucleases[a,b]

Compound	Splitting, %				
	A	B	C	D	E
GpU	77·5	84·0	73·5		—
GpC	61·0	68·0	57·0	85	78
GpA	76·0	74·0	74·0	—	—
GpG	76·0	74·5	70·0	—	—
ApU	57·0[c]	14·0[d]	16·0[d]	0	12[c]
ApA	62·0[c]	12·5[d]	14·0[d]	—	—
ApG	100·0[c]	3·0	6·0[d]	—	—
UpU	36·0[d]	14·5[d]	7·0[d]	—	—
UpC	43·5[c]	0	17·0[d]	0	9[d]
UpA	62·0[c]	15·0[d]	24·0[d]	—	—
UpG	16·0[c]	11·0[d]	16·0[d]	—	—
CpC	3·5[d]	4·0	3·0	0	9[d]
CpA	52·0[d]	23·5[d]	36·0[d]	—	—
CpG	23·0	18·5[d]	19·0[d]	—	—
(2′ → 5′)-UpA	34·0[d]	0	0	—	0

[a] If not stated otherwise, the experiments were performed with the (3′ → 5′)-isomers; [b] for symbols A—E see Table I; [c] higher values due to contamination with phosphodiesterase; [d] X > p and Y were the only products of XpY splitting.

5. The above mentioned finding prompted us to examine the influence of substitution at position 8 of the guanine moiety in guanosine 2′,3′-cyclic phosphate upon the response of the present enzymes. The 8-bromo (*XVII*) and 8-amino (*XVIII*) derivatives were found to be good substrates for the whole enzyme set while the 8-hydroxy (*XIX*) and 8-mercapto (*XX*) derivatives are bad substrates. Interpretation of these observations is rather difficult as it is believed that introduction of a bulky substituent into position 8 results in a conformational shift from *syn* to *anti*; the 8-bromo- and 8-mercaptoadenine oligonucleotides[23,24] exhibit opposite CD-characteristics. On the other hand, the energy barrier of the rotation of the base about the nucleoside linkage appears to be low enough in the purine series to be overcome by the energy gain in the formation of the enzyme-substrate complex. Thus, the inductive or mesomeric effect of the 8-substituent might be more important than its steric requirements. The resistance of the 8-hydroxy (*XIX*) and 8-mercapto (*XX*) derivative towards the enzymes might be caused by a negative effect of the N^7—H proton which might be involved in the corresponding 8-oxo or 8-thioxo forms of these derivatives. Such a situation is impossible in the case of the 8-amino (*XVIII*) or 8-bromo (*XVII*) derivatives which are good substrates. On the other hand, the lone electron pair on the N^7 nitrogen atom might also be essential in the base binding or competitive interaction. Such an assumption might be inferred from the resistance of the 7-deazainosine derivative *XXV* in which the N^7 atom is replaced by a CH group[25]. The stability of the tubercidine derivative *XIV* towards ribonuclease II (*Asp. clavatus*) is of a similar nature; this enzyme cleaves the adenosine derivative *VI* while the 7-deaza analogue *XIV* is resistant.



Rf = β-D-ribofuranoside 2′,3′-cyclic phosphate

6. The change at position 4′ of the ribose moiety in the lyxofuranoside *XXI* does not result in any qualitative change in the affinity to the enzymes. Thus, compound *XXI* is split both by ribonuclease T 1 (*cf.*[26]) and all the present guanyloribonucleases.

It may be thus concluded from the above points that both ribonuclease T 1 and the related ribonucleases of *Asp. clavatus*, *Act. aureoverticillatus*, and *Str. aureofaciens* can be classified as cyclising 2′-D-nucleotidotransferases specific for purine derivatives containing a —NH—CO— group at position N^1—C^6, and requiring the presence of the N^7-atom in the imidazole moiety.

The unexpected observation on the cleavage of adenosine 2',3'-cyclic phosphate (*VI*) with ribonuclease II of *Asp. clavatus* should favour a similar "purine specificity" of this enzyme as in the case of ribonuclease U 2 (*cf.*[17]). To examine this possibility, the presence of adenosine deaminase in the experimental enzyme preparation was eliminated by analysis of the cleavage product: adenosine 3'-phosphate was found to be the single product of the ribonuclease II treatment of adenosine 2',3'-cyclic phosphate and no inosine derivative was detected even in traces. Furthermore, the general characteristics of cyclic phosphates and 3'-nucleotides released by digestion of yeast ribonucleic acids resemble the pattern of the U 2 enzyme[7].

Ribonucleoside 2',3'-cyclic phosphates are known to be much worse substrates than polynucleotides or oligonucleotides. To obtain reliable data on the specificity and analytical applicability of the above enzymes, their response to dinucleoside phosphates was also examined. Table III shows the corresponding results obtained with the standard enzyme concentration as used in the study on ribonucleoside 2',3'-cyclic phosphates, *i.e.*, the concentration was always higher than necessary.

As expected, all the four 3'-guanylic acid derivatives, namely, GpU, GpC, GpA, and GpG are good substrates for the present enzymes. However, there has not been observed any increased activity of ribonuclease II from *Aspergillus clavatus* towards the 3'-adenylic acid derivatives ApA, ApG, and ApU; in all cases, the cleavage led to a mixture of adenosine 2',3'-cyclic phosphate and the nucleoside only without any appreciable formation of 3'-adenylic acid. Moreover, this cleavage was rather poor and did not even achieve the level of adenosine 2',3'-cyclic phosphate itself (Table II). The level of the cleavage was similar to that observed in the case of the *Aspergillus clavatus* ribonuclease C 2. The ribonuclease II of *Asp. clavatus* thus does not resemble the ribonuclease U 2 since the latter enzyme shows preference for adenylic acid esters[17]; moreover, the U 2 enzyme splits the internucleotidic linkages much better than 2',3'-cyclic phosphates in contrast to the former enzyme. Ribonuclease II would thus require a more detailed investigation, particularly with respect to its behaviour towards adenine nucleotides.

The general view on the behaviour of guanyloribonucleases towards natural internucleotidic derivatives as summarised in Table III, is rather striking. Taking into account the experimental conditions, the data more likely correspond to a preferential splitting than to a specific behaviour. Most of the 16 doublets investigated are degraded by most enzymes of the present set without any obvious dependence on structural features. In addition to 3'-guanylic acid dinucleoside phosphates which are perfect substrates, there is observed a significant cleavage of the internucleotidic linkage with uridylic, cytidylic, and adenylic acid dinucleoside phosphates by all the three *Aspergillus* enzymes. The two ribonucleases of *Aspergillus clavatus* produce solely the 3'-end nucleoside and the 2',3'-cyclic phosphate of uridine, cytidine or adenosine. The same general features were also observed in the case of the ribonuclease T 1 preparation; in the latter case, however, the results are unfortunately

affected by a high level of the contaminating phosphodiesterase activity. This contamination causing cleavage of ApG to 5'-GMP and adenosine and of ApU to 5'-UMP and adenosine, was present in two different samples of the commercial enzyme preparation. Nevertheless, the formation of ribonucleoside 2',3'-cyclic phosphates and nucleosides occurred to a very high extent in the presence of ribonuclease T 1. The possibility of a non-specific splitting of dinucleoside phosphates with ribonuclease T 1 has been mentioned by Egami and Uchida[17]. It may be inferred from our experiments shown in Table III that the non-specific reaction virtually takes place; the non-specific reaction cannot be due to any contamination with ribonuclease T 2 as suggested by Egami and Uchida[17] since the 2',3'-cyclic phosphates formed would be rapidly cleaved by the T 2 enzyme. The order $G \gg A > U > C$ suggested by the above authors might be accepted with some limitations; a detailed examination of the reaction would require kinetic analysis. The $(2' \rightarrow 5')$-isomer of UpA is split with ribonuclease T 1 to afford uridine 2',3'-cyclic phosphate and adenosine, *i.e.*, the same products as in the case of the natural $(3' \rightarrow 5')$-isomer. The reaction is consequently not specific for the 3'-nucleotide derivatives. Its mechanism obviously consists in activation of the phosphodiester linkage by a proper amino acid arrangement at the active site of the enzyme responsible for the binding of the normal substrates or at some other site of similar properties; the activation step is followed by an intramolecular attack of the neighbouring 2'- (or 3') hydroxylic function to afford the 2',3'-cyclic phosphodiester and to release the 3'-end nucleoside. This situation is reminiscent of the alkaline hydrolysis of the internucleotidic linkage. The single requirement of the reaction to take place consists in attachment of the diester molecule to the proper position on the enzyme for a sufficiently long period of time. The nucleotides of adenosine, uridine, and cytidine are known to inhibit ribonuclease T 1; consequently, they also must be bound to the enzyme[2]. Undoubtedly, the probability of the complex formation which would be of a sufficient life-time to accomplish the reaction, is increased by the cooperative interaction with the 3'-end nucleoside present. We failed to detect any formation of 2',3'-cyclic phosphates in the reaction of ribonuclease T 1 with ethyl esters of 3'-adenylic, 3'-uridylic, and 3'-cytidylic acid.

The non-specific reaction is substantially suppressed in the case of the *Asp. clavatus* ribonucleases and the *Streptomyces* ribonuclease. Concerning the *Actinomyces* enzyme, the level of the non-specific reaction lies within limits of experimental error. The reaction might be consequently due to the presence of a contaminant protein which asserts itself in view of the high concentration of the enzyme. Nevertheless, some features of the reaction favour this behaviour as the intrinsic property of the enzymes in question, namely, the non-specificity with respect to bases and the internucleotidic linkage and the overall similarity to an alkaline hydrolysis. Though obviously affected by contamination with a phosphodiesterase, the non-specific activity appears to be the highest in the case of ribonuclease T 1; this observation

must be born in mind in analytical applications. In this respect, the remaining other enzymes of the present set would be less questionable.

The evaluation of the above observations is complicated by a serious problem. For the sake of comparison of various similar enzymes, the activity is usually expressed in enzyme units. The analytical procedure is based on the hydrolysis of the high-molecular RNA and does not necessarily reflect the different affinity of enzymes towards the oligonucleotides formed in the first step. The assay thus mainly reflects the transfer reaction of the enzyme. A proper low-molecular standard substrate would be desirable in the hydrolytic reaction instead of RNA to allow a quantitative comparison of various enzymes.

In connection with the original aim of our investigation it may be concluded that the similarity of "specific" guanyloribonucleases from different microorganisms towards some modified substrate analogues is much deeper than that of "non-specific" enzymes[8]. The observed differences are rather of the quantitative than qualitative nature. Consequently, the requirements of the recognition or binding sites to chemical effects within the group of "specific" ribonucleases[10] are similar.

EXPERIMENTAL

Enzymes. Aspergillus clavatus extracellular ribonuclease C 2 (*cf.*[6]) and intracellular ribonuclease II (*cf.*[7]) as well as the *Actinomyces aureoverticillatus* ribonuclease[3] were prepared by procedures described earlier. Ribonuclease T 1 was a commercial preparation of Sankyo Co., Japan. Estimation of the enzymatic activity of ribonucleases was performed by a modified procedure[27] consisting in determination of acid-soluble products from the yeast RNA splitting by spectrophotometrical measurements at 260 nm.

Substrates. For references of the substrate preparations see an earlier paper[20]. 2'-O-Tetrahydropyranylguanylyl-(3'→5')-uridine was prepared by a procedure reported elsewhere[20] for the 3'-uridylic acid analogue.

Assay of Hydrolase Activity with Ribonucleoside 2',3'-Cyclic Phosphates

The incubation mixture contained 2 μmol of the substrate and 100 e.u. of the appropriate enzyme (50 e.u. *Str. aureofaciens* ribonuclease) in 50 μl of the 0·05M sodium β,β-dimethylglutarate buffer solution (pH 6). The blank control experiments were performed analogously, only the enzyme solution was replaced by an equal volume of the above buffer solution. Aliquots of the mixture were withdrawn in 4 and 24 h intervals (25 μl each) and chromatographed on a paper Whatman No 3 MM in the 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2) solvent system overnight. Spots of starting materials and products were detected under UV light, cut out, eluted with 10 ml portions of water, and the eluates measured after 24 h on a Beckmann DU apparatus at 260 nm. Values exceeding by 10% those of the blank experiment were taken as significant. The results are summarised in Table II.

Assay of Enzymatic Activity with Diribonucleoside Phosphates

The incubation mixture contained 1 μmol of the substrate and 50 e.u. of the appropriate enzyme in 50 μl of the 0·05M sodium β,β-dimethylglutarate buffer solution (pH 6·0). The blank control experiments were performed analogously, only the enzyme solutions were replaced by an equal volume of the above buffer solution. After 24 h at 37°C, the mixture was analysed as above. Spots of starting materials and products (3′-nucleotides or 2′,3′-cyclic phosphates and nucleosides) were eluted and the eluates measured as above. For the results see Table III.

Phosphodiesterase Assay in Ribonuclease T 1 Preparation

Spots of mononucleotides obtained by digestion of ApG and ApU with ribonuclease T 1 under the above conditions were eluted by a minimum volume of water. The UV spectra of eluates corresponded closely to those of guanylic and uridylic acid, resp. A portion of eluates (about 1 μmol) was treated with 10 μg of bacterial alkaline phosphatase (Worthington) in 50 μl of the 0·05M-Tris-HCl buffer solution (pH 9·0) at 37°C for 2 h. The mixtures were analysed as above. As UV-absorbing materials, only guanosine and uridine, resp., were present as proved by comparison with authentic specimens.

Analysis of the Splitting Product of Adenosine 2′,3′-Cyclic Phosphate with Ribonuclease II (*Asp. clavatus*)

Adenosine 2′,3′-cyclic phosphate was incubated with ribonuclease II as above. The spot corresponding to adenylic acid was eluted with a minimum volume of water. Paper electrophoresis in 0·05M sodium hydrogen citrate buffer solution (pH 3·5) on paper Whatman No 3 MM (40 V/cm, 1 h) indicated adenylic acid as the single material present as proved by comparison with an authentic specimen. Furthermore, the UV spectrum of the eluate corresponded to that of adenylic acid (the same concentration at pH 2). Finally, the analysis of the eluate by chromatography in saturated ammonium sulfate–1M sodium acetate–2-propanol (79 : 19 : 2) gave a single spot identical with that of 3′-adenylic acid. No trace of the 2′-isomer was detected.

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