MECHANISM AND BINDING SITES IN THE RIBONUCLEASE REACTION
II. KINETIC STUDIES ON THE FIRST STEP OF THE REACTION[1]

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It has been suggested that binding to the enzyme through the phosphodiester-monoanion occurs in both the first and second steps of the ribonuclease (RNase) reaction (1, 2). However, the velocity of the first-step reaction appears to vary very greatly, depending on the nature of the substrate, as shown by a qualitative comparison of the action of RNase on different dinucleoside-phosphates (1).

Kinetic information about the first-step reaction cannot be obtained by following the degradation of RNA as substrate. This reaction is very complex, since binding is influenced by the macromolecular nature of the substrate and since the observed reaction rate results from cleavage of different nucleotide diester bonds in RNA with different velocities (3). Examination of the degradation of the benzyl or alkyl esters of 3'-cytidylic acid as smaller substrates using paper chromatographic methods (4, 5, 6) does not have sufficient accuracy to permit calculations of K_m and k_3[4] values.

We present here kinetic data for the first step of the RNase reaction, measured on several diesters, particularly dinucleoside-3',5'-phosphates (CpA, etc.), using a spectrophotometric method similar to that used in the previous paper (2). The measurement is based on a spectral change during

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1, 2, 3, 4 See corresponding footnotes to Paper I of this series (2). Abbreviations not defined are as in Paper I.

the reaction, e.g.

$$\text{CpA} \xrightarrow[\text{(RNase)}]{\text{(step I)}} \text{cyclic Cp} + \text{A} \xrightarrow[\text{(RNase)}]{\text{(step II)}} \text{3' - Cp} + \text{A}$$

The absorption at 286 mμ of 3',5'-CpA is the same as the mixture of 3'-Cp and A (a hypochromicity effect can be neglected at that wavelength), but the absorption of the corresponding mixture of cyclic Cp and A is lower, caused by the shift of the spectrum to shorter wavelength in the cyclic diesters. If step I is fast enough, then during I we observe a decrease in absorption followed by an increase during step II to the initial values.

Experimental: Ribonuclease "D" was prepared as in paper I (2). 3'-Cp benzyl ester (Cp-benzyl) was prepared from 3'-Cp and phenyl-diazomethane (7). 3'-Cp methyl ester (Cp-methyl) was a gift of Dr. M. Irie. Dinucleoside-phosphates were prepared by hydrolysis of yeast RNA in the presence of bismuth hydroxide (8). After separation on a Dowex 1-X2 ion-exchange column they were, where necessary, finally purified by paper chromatography in isopropanol-ammonia-water (7:1:2). They were pure and free of the 2',5'- isomers, as shown by complete RNase hydrolysis.

The frozen-dried compounds were dissolved in 0.1 M imidazole buffer (pH 7.0), taken to an ionic strength of 0.2 with NaCl. Their concentrations were determined spectrophotometrically. In assays in the Cary spectrophotometer, as described in paper I, the decrease of the absorbance with time was recorded up to the end-point and, where required, the subsequent increase.

Initial velocities were determined from normal first-order plots. By using the first part of the reaction course (and the theoretical end-point) the reaction could be treated as independent of the second-step reaction, i.e. consecutive reaction theory was not required. K_m and k_3 were obtained by the Lineweaver-Burk method.

Results: Table I shows the results.

TABLE I

Kinetic constants at pH 7.0 (imidazole buffer), 26°

(K_m in M x 10^{-3}, k_3 in sec^{-1})

Substrate	k_3	K_m	Substrate	k_3	K_m
CpA	3000	1.0	UpA	1200	1.9
CpG	500	3.0	UpG	--	--
CpC	240	4.0	UpC	40	3.0
CpU	27	3.7	UpU	11	3.7
Cp (cyclic)	5.5	3.3	Up (cyclic)	2.2	5. 0
Cp-benzyl	2	3			
Cp-methyl	0.5 (from initial velocity, assuming K_m in the same range)				

With Cp-methyl the rate of the first step is so slow compared with the second step that no reasonable calculation was possible. Cp-benzyl, also, is slower in the first step and therefore shows a decrease in the absorbance to only 28% of the theoretical decrease, reaching a pseudo steady state near there; only the very initial portion (1 minute) could be used in deriving the initial velocities for this substrate. With UpU the steady state was reached after 85% decrease. The fastest substrates showed the theoretical decrease and subsequent return: the cyclic phosphates are therefore formed as obligatory intermediates.

The half-time of RNase hydrolysis of cyclic Cp formed during the reaction of CpA was found to be the same as in the reaction of cyclic Cp alone, or of cyclic Cpin equimolar mixture with adenosine.

Discussion: The K_m values for the first-step substrates do not differ from the values for the second-step substrates; only in the cases of CpA and UpA is K_m a little, but significantly, lower. An interpretation of these data

in terms of a common binding site in the enzyme and also in the substrates will be given elsewhere in connection with further results on the pH dependence of the kinetic constants and on inhibition in both steps.

In contrast, k_3 shows enormous differences, dependent on the nature of the second alcohol and particularly of the second nucleoside. The difference between the benzyl and methyl esters (the isopropyl ester reacts even more slowly than the methyl ester [5]) can be explained by the decreased electrophilicity at the phosphorus atom, corresponding to the "leaving tendency" of the alcohol group. The same reason may explain the higher value for the cyclic diester, although here additional differences exist in the nucleophilicity and position of the attacking 2'-OH group and the H_2O molecule. In the dinucleoside-phosphates the differences cannot be explained on the same basis, because the ester bond to the phosphorus is in all cases the same 5'-OH group of the ribose. Therefore, we have to assume that the second factor determining the velocity in the catalysis, namely the nucleophilicity of the pyrimidine base (2), is itself influenced by the second nucleoside. The enormous increase in nucleophilicity - the reaction rate is 6000-fold higher in CpA than in Cp-methyl under the conditions mentioned - can be explained only on the basis of a π-interaction between the pyrimidine base and the second base, increasing the polarisability and therefore the nucleophilicity of the catalysing system. In the position required for reaction (1, 2) this is sterically possible, as can be demonstrated on models.

The interpretation in the terms of π-interactions, and not a localised interaction (perhaps through the C-6-substituents), is based on the following facts: (1) the purines activate more strongly than the pyrimidines, which have a lower degree of aromaticity, especially uridine; (2) the differences cannot be explained by the known H-bonding affinities of the pairs concerned. The ratio of the rates for cyclic Cp and cyclic Up (2.5-3) is the same as that for CpA and UpA or for CpU and UpU, although in UpA an activation should occur by an H-bridge withdrawing the proton from the C-6-OH group, and in CpU an inhibition by the transfer of a proton to the C-6-NH_2 group. Thus

the contribution of the second base to the nucleophilicity of the pyrimidine base involved seems to be independent of the C-6-substituent. (The rather greater - 6-fold - difference between CpC and UpC is not sufficient to invalidate this argument); (3) a mixture of cyclic Cp and adenosine does not show acceleration of the second step reaction, although such an acceleration has now been observed in ApC-cyclic-p (i.e. adenylyl-(3',5')-cytidine 2',3'-phosphate); (4) there is evidence that a hypochromicity effect in the dinucleoside-phosphates is based on a similar π-interaction between the two bases, according to Michelson (9), and in general (although all cases have not been tested) the same series is usually shown for the influence of the second base: A > G > C > U (9, 10).

This interpretation in terms of π-interactions, increasing the polarisability at the nucleophilic centre, requires a mechanism of the type proposed (1, 2). In such a case, catalysis does not arise from a permanent increase in polarisation but from an increase of the amplitude of polarisability in the C-2'-OH bond, by joining the hydrogen to a highly resonating system, which can be amplified by other appropriate systems. A discussion and more examples of this type of catalysis will be given later.

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