MECHANISM AND BINDING SITES IN THE RIBONUCLEASE REACTION

I. KINETIC STUDIES ON THE SECOND STEP OF THE REACTION[1]

H. Witzel[2] and E. A. Barnard[3]

Virus Laboratory, University of California, Berkeley 4, California

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Pancreatic ribonuclease catalyses the conversion of 3'-pyrimidine-nucleoside-phosphodiesters to the corresponding 3'-phosphomonoesters in two steps (1, 2). In the first step the diesters are converted to the cyclic 2',3'-diesters by an intra-molecular attack of the 2'-oxygen, splitting the second ester bond. In the second step the cyclic diesters are hydrolysed by the attack of a water molecule, splitting only the 2'-ester bond. The requirements for the reaction appear to be a pyrimidine base with an intact mesomeric system $-\underset{\underset{O(S)}{\|}}{C}-N=C-CH(NR_2)$ (3), the 3'-position of the phosphate ester linkage (2) and a free phosphodiester monoanion (4).

The specificity for pyrimidine bases may be interpreted in terms of a specific binding to the enzyme (5, 6). A completely different explanation has been given by Witzel (7), in which a concerted reaction mechanism is invoked involving the assistance of the pyrimidine base in the catalysis with the following transition states for the first (I) and the second (II) step:

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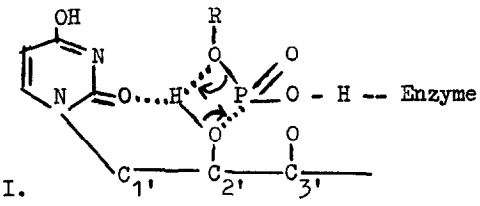
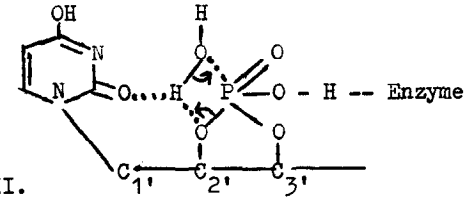
[1] Aided by a United States Public Health Training Grant, CRTY 5028

[2] On leave of absence from Chemisches Institut der Universität Marburg, Germany. Present address: Virus Laboratory, University of California, Berkeley.

[3] Fellow of the Rockefeller Foundation (1960-61), on leave of absence from King's College London, England (present address, after December 1961).



I.



II.

The velocity of the reaction should be determined by the nucleophilicity at the C-2-oxygen of the pyrimidine base and the electrophilicity at the phosphorus atom. In the purine analogues the steric situation is different (no suitable group in a purine is near enough to both the C-2'-O and the phosphate group) and, therefore, the base cannot play the same role in the catalytic process.

In this mechanism the binding of the diesters to the enzyme occurs through the $-P\begin{matrix}O\\ \diagup\\ \diagdown\\ O^-\end{matrix}$ group, and must be associated with an increase of the electrophilic character of the phosphorus atom. The binding itself, it is suggested, depends on the nucleophilicity of the $-P\begin{matrix}O\\ \diagup\\ \diagdown\\ O^-\end{matrix}$ anion.

We have sought to investigate the alternative situations, participation of the base either in substrate binding or in catalysis, by examining the kinetic behaviour of four different cyclic nucleotides, chosen so that the nucleophilicity of the phosphate anion differs only by small inductive effects, but with variations in the base such that a considerable change can be expected in the potential interacting site with regard to binding affinities or to catalytic activity.

The effects were examined by a kinetic analysis deriving K_m and k_3[4] at various pH values.

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[4] Using the scheme $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$, where $K_m = \frac{k_2 + k_3}{k_1}$, we assume that K_m is entirely or very largely a measure of the substrate binding in this reaction, although the relevant individual rate constants have not yet been determined. Evidence bearing on this point will be discussed elsewhere. However, arguments based on the relative magnitudes of k_3 values are independent of this assumption.

Experimental: Fraction "D" (8) was prepared from pancreatic ribonuclease (Armour) and dialyzed exhaustively (finally in 0.1M NaCl). 2',3'-cytidylic acid (Cp), 2',3'-uridylic acid (Up) and N^6-acetyl-2',3'-cytidylic acid were prepared by the method of Michelson (9) and purified by paper chromatography; 4,5-dihydro-2',3'-uridylic acid was prepared from the corresponding cytidylic acid by hydrogenation with rhodium on alumina (10) at pH 4 (adjusted with acetic acid). Complete deamination occurs during the reaction.

The enzyme reactions were followed by a modification of the UV spectral method (11a, b) using a Cary spectrophotometer, or by auto-titration on a Radiometer pH-stat (in 0.2 M NaCl). In the spectral assays, buffers were 0.1 M imidazole (pH 7.03) and 0.1 M acetate (lower pH), both taken to a total ionic strength of 0.2 with NaCl. The substrates were present in 1.2 ml buffer solution in a 1.5 ml cell (1 cm) of a Cary spectrophotometer equipped with a thermostat. The concentrations were determined spectrophotometrically. After adding enzyme solution (suitable dilutions in 0.2 M NaCl) and mixing (5 sec) the increase of absorbance with time was recorded up to the end-point, using 286 mμ for cyclic Cp, 275 mμ for cyclic Up, and 307 mμ for cyclic N^6-acetyl Cp, or a suitable longer wavelength, if the concentration was higher.

Initial velocities were determined from normal first-order plots, and K_m and k_3 determined from Lineweaver - Burk plots (12).

Results: The results are given in Table I. The values for K_m are in general lower than the values given by Herries (13) for cyclic Cp and agree with the single point of Hummel et al. (14). The k_3 values in the pH-stat measurements are higher than the values in imidazole buffer at pH 7, but agree with Herries' values obtained under the same conditions. The relative difference between cyclic Cp and cyclic N^6-acetyl-Cp is the same at pH 7 and 5.8 and does not appear to be pH-dependent.

Discussion: It can be expected that in N^6-acetyl-Cp the nucleophilicity at the pyrimidine-C-2-oxygen is reduced compared with Cp. Also hydrogen bonding

TABLE I

Kinetic Constants for Hydrolysis of Cyclic Phosphates at 27°

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

Substrate	pH 7.0 K_m	pH 7.0 k_3	pH 5.8 K_m	pH 5.8 k_3
Cyclic Cp	3.3	5.5 16 †	0.4	2.0
Cyclic N^6-acetyl-Cp	5.5	0.45	0.4	0.17
Cyclic Up	5.0	2.2 6 †	0.5	1.0
Cyclic dihydro-Up	5-7†	0.5†		
	pH 5.0		pH 4.0	
Cyclic Up	0.6	0.5	2.0	0.16

† pH-stat values (with accuracy lower than in the spectral measurements). All others are in buffers. K_m, unlike k_3, is not significantly changed in the presence of imidazole (pH 7).

through the NH_2-group or N' as an acceptor would be weakened. In dihydro-Up, as compared with Up, the resonating system is reduced, lowering the polarizability at the C-2-oxygen, but the basicity in this system (as determined by the pK_a-value of the C-6-OH group) is increased (15). Any contribution due to substrate binding to the enzyme through π-complex formation would be greatly disturbed by the loss of the pseudo-aromatic planar ring structure.

It is seen that the changes in the pyrimidine base cause only small changes in K_m, but k_3 values are changed considerably. It thus appears that binding through the pyrimidine base cannot play a major role in establishing

the specificity. The catalytic step, in contrast, is very strongly influenced by the change in the pyrimidine base, with particular reference to a change in its resonance structure or polarisability rather than to its basicity.

These results fit a mechanism in which the pyrimidine base is involved in the catalysis, as proposed in the mechanism of Witzel (7). Any mechanism invoked must be able to account for the dependence of k_3 upon the structure of the pyrimidine base.

If the pyrimidine base catalyses the reaction without being involved in the binding, then the pH-dependence curve of k_3 reflects (i) the change of nucleophilicity of the pyrimidine base with pH, which seems to be dependent on the nature of the buffer bases as well as on the absolute H-ion concentration, in addition to (ii) the amount of effective enzyme-substrate complex as determined by the ionising groups concerned in it. Under these circumstances k_3 does not depend simply on ionising groups on the enzyme, and deductions as to the nature of the interactions of substrate and enzyme, made from a normal type of analysis of the pH-dependence of k_3, and K_m, may not be justified.

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