The Mechanism of Action of Ribonuclease

By ANDREW WILLIAMS
(*Chemistry Department, University of Kent, Canterbury*)

THE purpose of this Communication is to prove the existence of a direct pathway, k_3, in the hydrolysis of cytidyl-3′,5′-cytidine catalysed by ribonuclease. Cyclic 2′,3′-phosphates occur during the ribonuclease-catalysed hydrolysis of small synthetic nucleotides and polynucleotides.[1–3] The formation of these cyclic intermediates was demonstrated by Witzel and Barnard[3] who observed two steps (k_1 and k_2); k_2 was identical with the rate constant for the ribonuclease-catalysed hydrolysis of cytidyl-2′,3′-phosphate.

The overall mechanism of hydrolysis of nucleotide diesters is an intramolecular attack of the 2′-hydroxyl group at the 3′-phosphate yielding a cyclic phosphate (step k_1) which reacts with water (step k_2), and both steps are catalysed by ribonuclease.[3–5]

The direct attack of water (step k_3) on cytidyl-3′,5′-cytidine in the enzymic site has not hitherto been contemplated as an alternative mode of hydrolysis.

Reactions k_1 and k_2 were followed spectrophotometrically[3] at 2860 Å; we found $k_1 = 7{\cdot}2 \times 10^4\,\mathrm{M}^{-1}\mathrm{sec.}^{-1}$ and $k_2 = 1{\cdot}57 \times 10^3\,\mathrm{M}^{-1}\mathrm{sec.}^{-1}$ (pH 7, 25°). The concentration of cyclic diester [II] formed *via* step k_1 was calculated from the change in the ultraviolet absorption corresponding to step k_2. It was much less than that expected from $k_1 = 46k_2$ and the initial concentration of cytidyl-2′3′-cytidine $[\mathrm{I}_0]$. The ratio $R = [\mathrm{II}]/[\mathrm{I}_0] - [\mathrm{II}]) = 6{\cdot}75$ was not affected by doubling $[\mathrm{I}_0]$. Thus an alternative mode of reaction exists with a rate constant $k_1/R = 10{\cdot}7 \times 10^3\,\mathrm{M}^{-1}\mathrm{sec.}^{-1}$ Since $[\mathrm{I}_0]$ was less than K'_m, $k_1 = k'_\mathrm{cat}/K'_\mathrm{m}$. It can be shown that, because R does not vary with $[\mathrm{I}_0]$ when $[\mathrm{I}_0]$ is less than K'_m, $[\mathrm{I}_0]$ is less than K'''_m and $k_3 = k'''_\mathrm{cat}/K'''_\mathrm{m}$.

$$\text{(I)} \xrightarrow{k_1 .\, E} \text{(II)} \xrightarrow{k_2 .\, E .\, H_2O} \text{(III)}$$

(cytidyl-3′,5′-cytidine) (cytidine-2′,3′-phosphate) (3′-cytidylic acid)

$$\text{(I)} \xrightarrow{k_3\, E .\, H_2O} \text{(III)}$$

[k'_{cat} and K'_{m}, k''_{cat} and K''_{m}, k'''_{cat} and K'''_{m} are the Michaelis constants for the steps k_1, k_2, and k_3 respectively.

Experiments with a pH-stat confirm the presence of an alternative pathway and show that this involves the direct production of acid. If the mechanism involves only steps k_1 and k_2 it can be shown from the spectrophotometric work that acid should only be liberated after an induction period of about 35 sec. after the addition of enzyme (to give $0{\cdot}4 \times 10^{-6}$M)—the k_1 step alone does not involve the formation of acid. Acid was produced from zero time and its production decelerated for approximately 30 sec. when the rate became zero order. A rate constant $k_3 \sim 3 \times 10^3$M^{-1}sec.$^{-1}$ (pH 7, 25°) was calculated from the slope of the initial portion of the pH-stat trace. The accuracy of the experiment does not allow a precise value for k_3; nevertheless, this does agree with the result of the spectrophotometric experiments.

3′-Cytidylic acid is a competitive inhibitor of the k_2 step;[6] at $1{\cdot}8 \times 10^{-4}$M this reduces k_1 and k_2 1·6-fold. R is not affected so that k_3 is also reduced 1·6-fold and it is probable that all the steps occur at the same enzymic site.

The 2′-hydroxyl group reacts intramolecularly 6·75 times more efficiently than the intermolecular action of 55 molar water. The molarity must be 370 before the water can compete on equal terms with the 2′-hydroxyl group if there is no water-binding site. This is compatible with other comparisons of intramolecular with intermolecular processes.[7]

(*Received, July 27th,* 1966; *Com.* 547.)

---

[1] R. Markham and J. D. Smith, *Biochem. J.*, 1952, **52**, 552.

[2] D. M. Brown, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.*, 1952, 2715.

[3] H. Witzel and E. A. Barnard, *Biochem. Biophys. Res. Comm.*, 1962, **7**, 295.

[4] D. Findlay, D. G. Herries, A. P. Mathias, B. R. Rabin, and C. A. Ross, *Biochem. J.*, 1962, **85**, 152.

[5] H. A. Scheraga and J. A. Rupley, *Adv. Enzymol.*, 1962, **24**, 161.

[6] D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, 1962, **85**, 127.

[7] T. C. Bruice, *Brookhaven Symposia in Biology*, 1962, **15**, 52; M. L. Bender and M. C. Neveu, *J. Amer. Chem. Soc.*, 1958, **80**, 5388; B. Capon, *Quart. Rev.*, 1964, **18**, 45.