

## The Mechanism of Action of Ribonuclease

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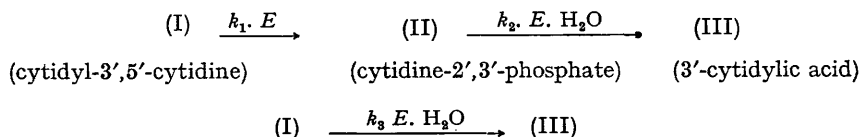
THE purpose of this Communication is to prove the existence of a direct pathway,  $k_3$ , in the hydrolysis of cytidyl-3',5'-cytidine catalysed by ribonuclease. Cyclic 2',3'-phosphates occur during the ribonuclease-catalysed hydrolysis of small synthetic nucleotides and polynucleotides.<sup>1-3</sup> The formation of these cyclic intermediates was demonstrated by Witzel and Barnard<sup>3</sup> 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.<sup>3-5</sup>

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<sup>3</sup> at 2860 Å; we found  $k_1 = 7.2 \times 10^4 \text{ M}^{-1}\text{sec.}^{-1}$  and  $k_2 = 1.57 \times 10^3 \text{ M}^{-1}\text{sec.}^{-1}$  (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  $[I_0]$ . The ratio  $R = [II]/[I_0] - [II] = 6.75$  was not affected by doubling  $[I_0]$ . Thus an alternative mode of reaction exists with a rate constant  $k_1/R = 10.7 \times 10^3 \text{ M}^{-1}\text{sec.}^{-1}$ . Since  $[I_0]$  was less than  $K'_m$ ,  $k_1 = k'_{cat}/K'_m$ . It can be shown that, because  $R$  does not vary with  $[I_0]$  when  $[I_0]$  is less than  $K'_m$ ,  $[I_0]$  is less than  $K'''_m$  and  $k_3 = k'''_{cat}/K'''_m$ .



[ $k'_{\text{cat}}$  and  $K'_m$ ,  $k''_{\text{cat}}$  and  $K''_m$ ,  $k'''_{\text{cat}}$  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  $0.4 \times 10^{-6}\text{M}$ )—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  $k_3 \sim 3 \times 10^3 \text{M}^{-1} \text{sec}^{-1}$  (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;<sup>6</sup> at  $1.8 \times 10^{-4}\text{M}$  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.<sup>7</sup>

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