URIDINE 2',3'-*O,O*-CYCLOPHOSPHOROTHIOATE AS SUBSTRATE FOR PANCREATIC RIBONUCLEASE (I)

F. ECKSTEIN

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Germany

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Recently we have synthesized uridine 2',3'-*O,O*-cyclophosphorothioate [1] (U-2',3'-cyclic pS) in the hope that it might turn out to be a suitable substrate for pancreatic ribonuclease which could shed some more light on the mechanism of hydrolysis of this enzyme. In particular, it should help to answer the question whether there exists a pentacovalent phosphorus intermediate and if so, whether it pseudo-rotates during hydrolysis. The results reported below show that although a pentacovalent phosphorus intermediate cannot be ruled out, pseudo-rotation clearly does not occur.

U-2',3'-cyclic pS contains an asymmetric phosphorus atom which is bound to an optically active ribose. It is therefore a mixture of diastereoisomers. We have been able to separate these two isomers by crystallization of the triethylammonium salts. Isomer I, mp 197–201° (EtOH), ^{31}P-NMR: δ = 168 ppm (20% aq. EtOH, standard 30% H_3PO_4); isomer II, undistillable liquid, δ = 171 ppm.

The determination of the absolute configuration of isomer I by X-ray analysis is under way.

The apparent first order rate constants for acid as well as alkaline hydrolysis of both isomers are indistinguishable and are summarized in table 1. The data for the hydrolysis of uridine 2',3'-cyclophosphate (U-2',3'-cyclic p) are given for comparison.

The proton catalyzed hydrolysis is second order in H^+-concentration and, as reported elsewhere [1], is accompanied by a large exchange of sulfur by oxygen whereas the alkaline hydrolysis proceeds without such exchange. This result is in agreement with a pentacovalent phosphorus intermediate in the acid hydrolysis [2]. Whereas the alkaline hydrolysis of U-2',3'-cyclic pS is slower than the hydrolysis of U-2',3'-cyclic p by a factor of 6, the acid hydrolysis is slower by a factor of approximately 200.

Table 1
Apparent first order rate constants k * for non-enzymatic hydrolysis.

	0.15 N $HClO_4$	0.25 N KOH
U-2',3'-cyclic p	2.3	1.2
U-2',3'-cyclic pS	0.011	0.2

* In 10^{-3} sec^{-1} at 23°C, I = 1 by addition of NaCl; measured by recording the increase of absorption at 283 mμ.

The kinetics of the RNase catalyzed hydrolysis, however, show marked differences for the two isomers as indicated in table 2.

Table 2
Enzymatic hydrolysis †

	K_m (M)	k_{+2} (sec^{-1})
U-2',3'-cyclic p	6.2×10^{-3}	2.5
U-2',3'-cyclic pS isomer I	6.2×10^{-3}	0.5
U-2',3'-cyclic pS isomer II	50×10^{-3}	0.5

† Experiments were carried out essentially as described by Gassen and Witzel [3] in a Cary 15 spectrophotometer following the increase of absorption at 283 mμ at 25°. Initial velocities were determined by a first order rate plot, K_m- and k_{+2}-values by a Lineweaver-Burk plot.

The data show that the K_m-value of isomer I is

identical with the one of U-2′,3′-cyclic p but that its k_{+2}-value is lower by a factor of 5. This difference is almost the same as the one found for the rates in the alkaline hydrolysis. Isomer II is bound much more weakly by the enzyme but its k_{+2}-value is identical with the one of isomer I. The identity of the K_m-values for U-2′,3′-cyclic p and isomer I on the one hand and the difference of this value with the K_m-value for isomer II on the other indicate that in isomer I the sulfur does not primarily take part in the binding to the enzyme, whereas in isomer II the sulfur interferes considerably with the binding process. This problem will be discussed in more detail in a subsequent publication when we will have established the absolute configuration of the isomers.

To determine the inhibitory effect of uridine 3′-*O*-phosphorothioate (3′-UMPS) we followed the formation of C-2′,3′-cyclic p from CpA with pancreatic ribonuclease spectrophotometrically in the presence of 3′-UMP as well as 3′-UMPS. Both compounds show an identical K_i-value (0.95×10^{-3} M). Here, the sulfur apparently does not interfere with the formation of the enzyme-inhibitor complex.

Most interesting is the finding that during enzymatic hydrolysis of either isomer there is no exchange of sulfur as shown by using ^{35}S-labelled material. Because this result is obtained with both isomers the lack of exchange cannot be due to a binding of the substrate to the enzyme through sulfur in such a way as to prevent the exchange of sulfur. Since the hydrolysis of the five-membered ring could proceed without pseudorotation of a pentacovalent phosphorus intermediate [2] this result does not exclude the existence of such an intermediate, but it clearly shows that there is *no pseudo-rotation* of a pentacovalent phosphorus intermediate.

Hoping that such a pentacovalent phosphorus intermediate, if it existed at all, would be nucleophilic enough to react with 5,5′-dithiobis-(2-nitrobenzoic acid) [4] we added this reagent to the incubation solution. We could not, however, detect any increase of absorption at 413 mμ except for a reaction, after a lag phase of approximately 30 sec with the hydrolysis product, 3′-UMPS.

Further studies with U-2′,3′-cyclic pS on the mechanism of hydrolysis of this enzyme are under way.

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