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# HYDROLYSIS OF RIBONUCLEOSIDE 2′, 3′-CYCLIC PHOSPHATES BY INFLUENZA AND NEWCASTLE DISEASE VIRUSES

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*Summary.* — Two enzymatic activities hydrolysing ribonucleoside 2′, 3′-cyclic phosphates (2′, 3′-cNMP) to 2′- or 3′- nucleoside monophosphate were found associated with influenza and Newcastle disease viruses. The two enzymatic activities differed from each other by temperature optima and thermoresistance. 2′, 3′-Cyclic nucleotide 3′-phosphohydrolase was responsible for splitting of the substrate to 2′-NMP. Splitting of the substrate to 3′-NMP was due either to ribonuclease or to 2′, 3′-cyclic nucleotide 2′-phosphohydrolase.

*Key words: enveloped viruses; virus-associated enzymatic activities*

## *Introduction*

While passing through the cell membrane, enveloped RNA viruses take along on their surface some components of the cell membrane. Purified preparations of enveloped RNA viruses thus display a variety of nucleolytic activities, like ribonuclease, deoxyribonuclease, phosphatase and phosphodiesterase (Rosenbergová *et al.*, 1965; Trávníček and Říman, 1966; Rosenbergová and Pristašová, 1972; Hung, 1973; Oxford, 1973; Wiegers and Drzeniek, 1973; Pristašová and Rosenbergová, 1974; Arora *et al.*, 1976).

We are reporting results indicating that purified influenza and Newcastle disease viruses possess a high activity of decyclizing 2′, 3′-phosphodiesterases and describe some properties of these enzymes.

## *Materials and Methods*

Influenza virus A/Singapore/1/57 (H2N2) and Newcastle disease virus (NDV) strain Kansas were propagated in chick embryos as described (Rosenbergová and Pristašová, 1972) and purified by differential centrifugation and sedimentation in discontinuous and continuous density gradients (Rosenbergová *et al.*, 1981).

*Enzymatic reactions.* Ribonucleoside 2′, 3′-cyclic phosphates derived from uridine (cUMP), cytidine (cCMP), adenosine (cAMP) and guanosine (cGMP) were used as substrates. They were kindly supplied by Dr. A. Holý, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The activity of decyclizing 2′, 3′-phosphodiesterase was determined in a total volume of 0.2 ml containing 150—200 μg substrate and 100—200 μg virus protein. The reaction proceeded in the presence of 0.05 M Tris.HCl buffer, pH 7.2. After incubation for 2 hr

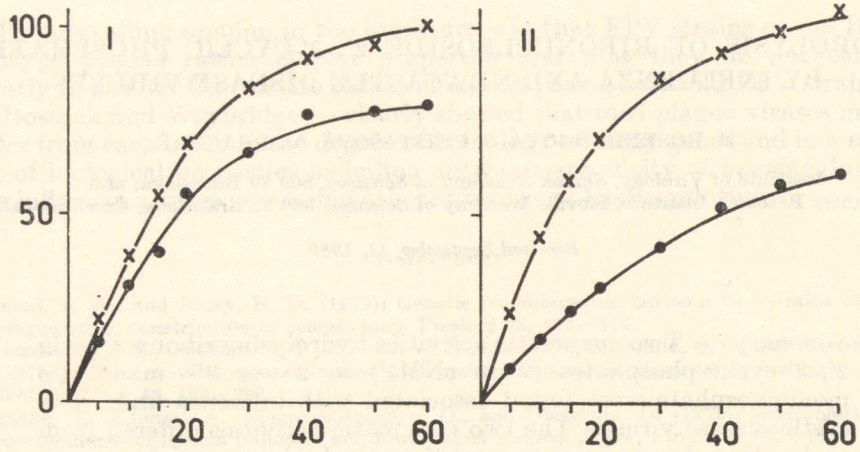


**Fig. 1.**
Kinetics of 2′, 3′-cAMP (I) and 2′, 3′-cCMP (II) hydrolysis at 60 °C

● Influenza virus, × NDV
Abscissa: time (min); ordinate: amount of NMP in %

at 30 or 60 °C, the reaction was stopped by heating in a boilling water bath. Proteins were removed by extraction with 2—3 volumes of chloroform and centrifugation for 20 min at 6000 × g.

The reaction products were determined by chromatography in the system 1-butanol: ethanol : : water (312 : 198 : 90 by volume) on Whatman No. 1 paper. The spots of enzymatic products were localized under ultraviolet light, cut out and eluted into 3 ml of 0.1 N HCl for 48 hr. Absorbance of the solutions was measured at 260 nm (280 nm with cytidine derivatives).

Protein contents were determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. The haemagglutinating (HA) activity of viral preparations was estimated by agglutination of 0.5% chick erythrocytes.

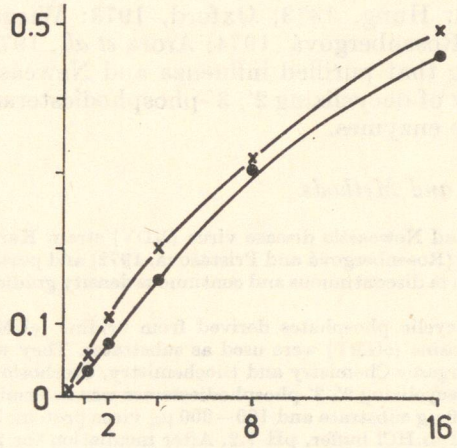


**Fig. 2.**
Dependence of 2′, 3′-cAMP hydrolysis on the concentration of influenza virus (●) and NDV (×)
Abscissa: virus concentration (HA units × $10^{-2}$); ordinate: AMP absorbance at 257 nm

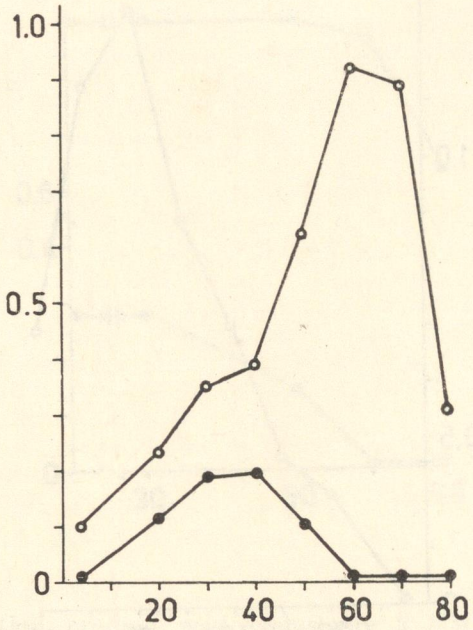


**Fig. 3.**

Temperature dependence of 2′, 3′-cAMP hydrolysis
○ 3′-AMP ● 2′-AMP
Abscissa: temperature of the enzymatic reaction (°C); ordinate: absorbance at 257 nm

*Results*

Both influenza virus and NDV splitted all four "natural" 2′, 3′-cNMP used to nucleoside monophosphates (NMP). Fig. 1 illustrates the time dependence of the hydrolysis of purine and pyrimidine 2′, 3′-cNMP. The amount of NMP hydrolysed increased in the course of 60-min incubation. With 3200 HA units of virus, the course of the 2′, 3′-cAMP hydrolysis curve was linear up to 10 min of incubation (influenza virus) or 5 min of incubation (NDV). With 2′, 3′-cCMP used as substrate the reaction was linear up to 15 and 10 min, respectively. In this case the amount of substrate hydrolysed by influenza virus was one-half that hydrolysed by NDV.

The concentration dependence of the enzymatic activities of the two viruses was studied on 2′, 3′-cAMP as substrate (Fig. 2). The amount of hydrolysed 2′, 3′-cAMP was a linear function of virus concentration under the standard incubation conditions.

In studying the temperature optimum of the reaction of decyclizing 2′, 3′-phosphodiesterase we separated the resulting product (NMP) to 3′- and 2′- isomers. The temperature optimum of the reaction was determined based on the maximum amount of the product. Fig. 3 illustrates hydrolysis of 2′, 3′-cAMP by NDV. At low temperatures, both 2′- and 3′-AMP were obtained but at high temperatures the only reaction product was 3′-AMP. Maximal hydrolysis to 2′-AMP was reached at 30—40 °C (first temperature optimum). Maximal hydrolysis to 3′-AMP occurred at 60 °C (second temperature optimum). Similar results were obtained with 2′, 3′-cGMP used as

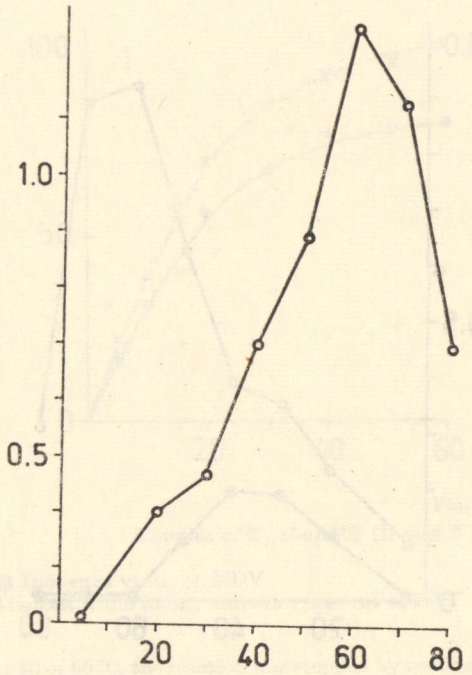


**Fig. 4.**

Temperature dependence of 2′, 3′-cCMP hydrolysis
Abscissa and ordinate as in Fig. 3.

substrate. As distinct from purine derivatives, 2′, 3′-cCMP was hydrolysed practically only to 3′-CMP (Fig. 4). At low incubation temperatures, 2′-isomers were formed in amounts at the limits of detectability by the method used and the total amount of hydrolysis products at these temperatures was much lower than with purine derivatives.

There was a marked difference in the thermostability of the enzymatic activities responsible for the formation of 2′- 2′- and 3′-NMP. Purified NDV was pre-heated for 1 hr at 30, 50 and 60 °C or for 15 min at 70 °C. The enzymatic reaction proceeded at the temperature optima for hydrolysis of the cyclic substrate to 2′-NMP (30 °C) and 3′-NMP (60 °C). The activity responsible for 2′-AMP formation proved to be thermolabile (Fig. 5). Heating at 50 °C resulted in a 50% loss of activity and heating at 60 °C caused complete inactivation of the enzyme. The activity responsible for 3′-AMP formation was thermoresistant and was not affected by heating for 1 hr at 60 °C.

## *Discussion*

Influenza virus and NDV possess decyclizing phosphodiesterase activities hydrolysing 2′, 3′-cNMP to nucleoside monophosphates. Depending on the temperature of the enzymatic reaction, two different isomers were formed.

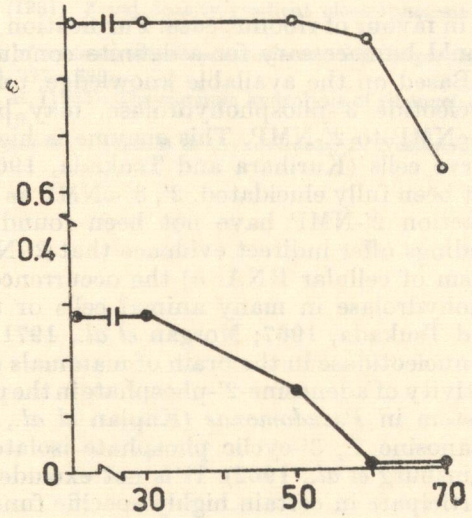


**Fig. 5.**

Thermal inactivation of NDV-associated decyclizing 2′, 3′-phosphodiesterase
○ 3′-AMP ● 2′-AMP
Abscissa: inactivation temperature (°C); ordinade: absorbance at 257 nm

At higher temperatures, the reaction product was exclusively 3′-NMP; maximum of its hydrolysis was at 60 °C. At lower temperatures, purine 2′, 3′-cNMP were hydrolysed to both 3′-NMP and 2′-NMP. Optimal hydrolysis to 2′-NMP occurred at 30 °C. These results indicate that the viral preparations contained two different enzymes hydrolysing 2′, 3′-cNMP. The enzyme responsible for the formation of 3′-NMP hydrolysed all four substrates used at an approximately same speed. By contrast, the enzyme responsible for 2′-NMP formation hydrolysed mainly purine cNMP. Pyrimidine 2′-NMP occurred only in trace amounts. The two enzymatic activities differed from each other in their resistance to heating. The activity yielding 2′-NMP was completely inactivated by heating for 1 hr at 60 °C while that yielding 3′-NMP was fully preserved under these conditions.

Hydrolysis of 2′, 3′-cNMP to a 3′-NMP could be catalysed in the cell by two enzymes: 2′, 3′-cyclic nucleotide 2′-phosphohydrolase or ribonuclease of the type of transfer ribonucleases which hydrolyse RNA over an intermediate 2′, 3′-cyclic phosphodiester further exclusively to 3′-isomers. Decyclizing phosphodiesterase of the type described above was demonstrated mainly in bacterial and plant cells (Rodden and Scocca, 1972; Holý and Rosenberg, 1978). In animals they were found in calf intestinal mucosa and in *Crotalus adamanteus* venom (Whitfeld *et al.*, 1955). Since the viruses studied were found to display a high ribonuclease activity (Rosenbergová and Pristašová, 1972) it cannot yet be concluded which type of enzyme associated with influenza virus and NDV is responsible for hydrolysis of 2′, 3′-cNMP to 3′-NMP. Similar thermal inactivation and temperature optimum of the reaction in hydrolysis of cNMP and high molecular weight RNA seems to

be in favour of ribonuclease. Purification of the enzymatic activity (activities) would be necessary for a definite conclusion.

Based on the available knowledge, only one enzyme, namely 2′, 3′-cyclic nucleotide 3′-phosphohydrolase, may be responsible for hydrolysis of 2′, 3′-cNMP to 2′-NMP. This enzyme is highly active in plasma membranes of nerve cells (Kurihara and Tsukada, 1967) and its role in the cells has not yet been fully elucidated. 2′, 3′-cNMP as well as the product of the enzymatic reaction 2′-NMP have not been found in animal cells but the following findings offer indirect evidence that 2′-NMP could participate in the metabolism of cellular RNA: *a*) the occurrence of 2′, 3′-cyclic nucleotide 3′-phosphohydrolase in many animal cells or their plasma membranes (Kurihara and Tsukada, 1967; Morgan *et al.*, 1971); *b*) the finding of a highly specific 2′-nucleotidase in the brain of mammals (Nakamura *et al.*, 1979); *c*) biological activity of adenosine-2′-phosphate in the pyridine nucleotide transhydrogenase system in *Pseudomonas* (Kaplan *el al.*, 1953); and *d*) the high amount of guanosine 2′, 3′-cyclic phosphate isolated from *Chromobacterium violaceum* (Ginsburg *et al.*, 1962). It is not excluded, however, that this enzyme could participate in certain highly specific functions.

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