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CLEAVAGE OF MOUSE RIBOSOMAL RNAs BY THE ENDONUCLEASE ASSOCIATED WITH NEWCASTLE DISEASE VIRUS

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Summary. — Mouse ribosomal ribonucleic acids (rRNAs) are specifically cleaved to polynucleotides of lower molecular mass by the endonuclease associated with Newcastle disease virus (NDV). The 28 S RNA yielded a fragment of about Mr $= 1.7 \times 10^5$ which is resistant to the endonuclease. We assume that one molecule of 28 S RNA contains one such resistant region. 18 S RNA does not possess resistant regions of this character and it is cleaved by the endonuclease to smaller molecules.

Key words: endonuclease; ribosomal RNA cleavage; Newcastle disease virus; polyacrylamide gel electrophoresis

Introduction

Enzymes degrading high molecular weight RNAs to polymers of smaller size were found associated with various viruses, e.g. avian myeloblastosis virus, NDV, influenza, vaccinia virus, foot and mouth disease virus. The activity of these enzymes was tested either with viral (Trávníček and Říman, 1966; Palese and Koch, 1972; Wiegers and Drzeniek, 1973; Denoya *et al.*, 1978; Paoletti and Lipinkas, 1978) or cellular rRNAs (Oxford, 1973; Storer *et al.*, 1973; Arora *et al.*, 1976). It was shown in our previous reports (Rosenbergová *et al.*, 1965; Rosenbergová *et al.*, 1971) that the endonuclease associated with viruses of avian myeloblastosis, influenza and NDV degrades chick rRNA to fragments of the size of about 5 S. The mammalian rRNA is cleaved in the same reaction to 8—10 S fragments.

In the present work we analyse degradation products of rRNAs from Ehrlich ascitic cells after the action of the endonuclease associated with NDV in the polyacrylamide gel electrophoresis.

Materials and Methods

Cellular RNA from Ehrlich ascitic cells. Cells were several times washed with saline and resuspended in 10 mmol/l Tris-HCl buffer pH 7.2, containing 1 mmol/l EDTA, 1% beta-mercaptoethanol and bentonite (1 mg/ml). The RNA was extracted three times with the buffer-saturated phenol, twice precipitated with ethanol in the presence of 120 mmol/l sodium acetate, and redissolved in TNE buffer. The TNE buffer contained 60 mmol/l NaCl, 10 mmol/l Tris-HCl and 2 mmol/l EDTA, pH 7.4.

rRNA from Ehrlich ascitic cells. It was prepared from the cellular RNA by centrifugation in a linear 5—20% sucrose gradient at 45 000 rev/min for 3 hr. The fractions, containing rRNAs were pooled, ethanol-precipitated and redissolved in TNE buffer. If pure 28 S and 18 S RNAs were isolated, only appropriate fractions corresponding to peaks of these rRNAs were pooled separately, ethanol-precipitated and redissolved in TNE buffer.

In some experiments, rRNA was treated with 2.5% diethylpyrocarbonate (DEPC) to prevent the action of nuclease during preparation. The sedimentation pattern of DEPC-treated rRNA showed no degradation, but there was a small peak faster than 28 S rRNA which could represent an aggregation.

^{3}H-uridine labelled 28 S RNA from L cells. Cultures of L cells in Basal Eagle Medium supplemented with 5% calf serum were incubated with ^{3}H-uridine (0.8 MBq per ml; specific activity 742 GBq per mMol) for 16 hr. 28 S RNA was isolated by phenol extraction and sucrose density gradient centrifugation as described above.

The RNA degradation. The reaction mixture (total volume 0.2 ml) consisted of 50—100 μg of RNA, 60—100 μg of viral protein in TNE buffer with 6 mol/l urea (TNE-urea buffer). The reaction proceeded at 37 °C for two hr unless otherwise specified and then it was stopped by addition of 50 μl of water-saturated chloroform. After a vigorous shaking the mixture was centrifuged at low speed, the clear water phase taken and supplemented with 20 μl of 1 mmol/l bromphenol blue in 8 mol/l urea. 100 μl aliquots were subjected to electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was carried out essentially according to Loening (1967), the concentration of acrylamide being 4.5% and that of bis-acrylamide 0.12% (w/w). The gels were made up in Tris-acetate buffer pH 7.6, containing 40 mmol/l Tris, 20 mmol/l sodium acetate, 2 mmol/l EDTA and 6 mol/l urea. The electrophoresis was carried out in Tris-acetate buffer without urea at 5 mA/gel for 75 min at room temperature. After the end of the run, gels were stained with 0.5% pyronin Y in acetic acid-methanol-water (1 : 1 : 8) for 16 hr according to Marcinka (1972). The absorbance of the stained material was measured with the Joyce-Loebl Chromoscan densitometer.

Determination of radioactivity of RNA in polyacrylamide gel. Gels after electrophoresis were frozen and cut into 2 mm slices. These were put into scintillation vials containing 1 ml of the mixture of 15% H_2O_2 and 0.1% NH_3, shaken for 4 hr at room temperature and dried overnight at 50 °C. Then 0.5 ml water and 6 ml of dioxan-naphtalene scintilation cocktail were added to the vials and their radioactivity was counted in Packard Tricarb Spectrometer Model 3390.

Molecular mass estimation. Molecular mass of the RNA fragment was estimated by meniscus depletion sedimentation equilibrium method accoring to Schachman (1963) using analytical ultracentrifuge Beckman Model E. The estimations were performed by courtesy of Dr. L. Horváth, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava. RNA dissolved in 10 mmol/l Tris-HCl and 100 mmol/l NaCl, pH 7.2 was centrifuged to equilibrium at 12 000 and 15 000 rev/min, respectively at 14.4 °C. The absorbance profiles at 260 nm were recorded by the photoelectric scanner. The buyoant density of RNA was estimated by isopycnic density gradient centrifugation in Cs_2SO_4 according to Nandi *et al.* (1965).

Propagation and purification of NDV. NDV-L strain Kansas was propagated in 10-day-old embryonated eggs. After 46 hr of incubation at 35 °C, the allantoic fluid was harvested, filtered through glass wool and clarified by centrifugation at 10 000 g for 15 min. The virus was then pelleted from the supernatant by high-speed centrifugation at 40 000 g for 1 hr and resuspended in TNE buffer. It was then centrifuged through a linear 20—55% (w/w) sucrose gradient in TNE buffer at 190 000 g for 4 hr. Virus zone was collected, pelleted by centrifugation at 40 000 g for 1 hr, resuspended in TNE buffer and further purified by centrifugation through a discontinuous 25—50% sucrose gradient at 40 000 g for 1 hr. Virus zone was again collected, pelleted by centrifugation at 40 000 g for 1 hr and resuspended in TNE buffer containing 0.05% sodium azide. The virus suspension was adjusted to the concentration of 3 mg protein per ml. The virus purified in this way and showing titres of $5—7 \times 10^5$ haemagglutination units per ml was used as the source of the enzyme.

Results

Analysis of the integrity of rRNAs

To eliminate possible artifacts in studying the cleavage products of the enzymic reaction, we attempted to unmask potential breaks in rRNA molecules

by their treatment with urea and to exclude a possible contamination of rRNA preparations with cellular nucleases by their treatment with DEPC. These control experiments on the integrity of our rRNA preparations were carried out under similar conditions as the reaction of enzymic degradation

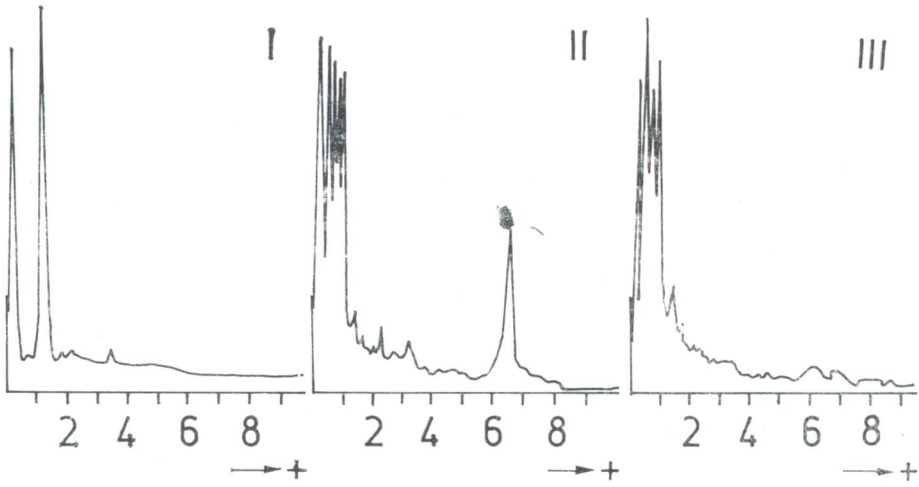

Fig. 1.

Effect of urea treatment on the electrophoretic mobility of rRNA from Ehrlich ascitic cells The PAG electrophoretic profiles. 40 μg of RNA was electrophoresed in 4.5% acrylamide-urea gels at room temperature and 5 mA/gel. I — rRNA incubated in TNE buffer pH 7.4, II — rRNA incubated in TNE-urea buffer pH 7.4, III — rRNA isolated from sucrose gradient after 10 min treatment with 6 mol/l urea at 90 °C and subsequent incubation in TNE-urea buffer. Abscissa: migration distance in cm

of rRNA. The rRNA from Ehrlich ascitic cells after 2 hr incubation in TNE buffer at 37 °C was separated in PAGE in such a manner, that 28 S RNA remains at the top of the gel and 18 S RNA migrates about 1 cm from the top (Fig. 1-I). If rRNA is incubated in TNE-urea buffer for 2 hr at 37 °C and electrophoresed, a larger amount of RNA migrates into the gel and several bands slower that 18 S RNA can be seen at the upper part of the gel. Moreover, a fast migrating fraction is observed (Fig. 1-II). The latter fraction can be eliminated, if rRNA is heated to 90 °C for 10 min in TNE-urea buffer, cooled and centrifuged in a linear 5—20% sucrose gradient in TNE-urea buffer (Fig. 2). The sedimentation profile of the urea-treated rRNA is markedly changed, so that most of 28 S RNA sedimented in the region of 18 S. On the other hand, a degradation to molecules smaller than 5 S, accumulating at the top of the gradient was avoided. The 28 S and 18 S RNA were isolated from the gradient by ethanol precipitation. The urea-treated rRNA is free of the fast migrating fraction in electrophoresis (Fig. 1-III).

The treatment of rRNA with DEPC with subsequent incubation in TNE-urea buffer does not influence its behaviour in electrophoresis, and the migration

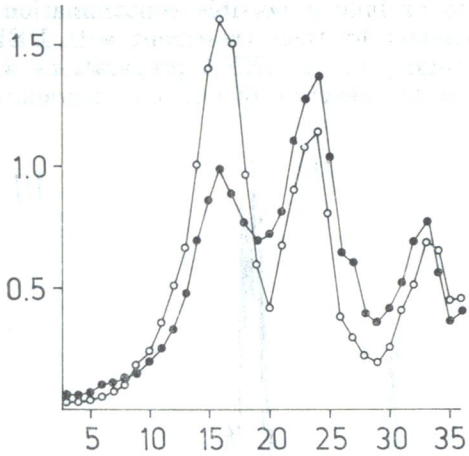

Fig. 2.
Sedimentation pattern of cellular RNA from Ehrlich ascitic cells in linear 5—20% (w/w) sucrose gradient in TNE-urea buffer.
●———● RNA heated 10 min at 90 °C in TNE-urea buffer.
⊙———⊙ not-heated RNA. Sedimentation from left to right.
Abscissa: fraction number
Ordinate: absorbance at 260 nm

profile is similar to that shown in Fig. 1. The results of these experiments support the view that the appearance of fast migrating fraction of RNA after the urea treatment is rather due to unmasking of hidden breaks in rRNAs, than to a cleavage of phosphodiester bonds during the enzymic reaction.

Cleavage of rRNA with the NDV-associated enzyme

Since as a source of the enzyme in our experiments we used NDV which contains high molecular mass genomic RNA, we had to check whether during the elctrophoresis of the products of the enzymic reaction a part of the viral RNA could not migrate in the gel. Therefore, NDV was incubated in the presence of 6 mol/l urea, treated with chloroform and electrophoresed. Fig. 3-I shows a single distinct peak situated about 1—2 mm below the top of the gel. This material was not further analysed and it may represent the viral nucleoprotein partially degraded during the deproteinization of the reaction mixture with chloroform. In the case that chloroform treatment was omitted, all the viral material would remain at the top of the gel after electrophoresis.

The electrophoretic profile of 28 S RNA, which was used in enzymic reaction as substrate, is shown in Fig. 3-II. Most of RNA does not enter the gel and only a small part of the material can be detected in two fractions in the upper part of the gel.

28 S RNA incubated with 60 μg of viral material in TNE buffer at 37 °C was degraded to a high molecular mass product, which migrated in electrophoresis as a distinct fraction about 3.5 cm from the start (Fig. 3-III). Other products of lower molecular mass yielded diffuse bands. If the enzymic reaction was carried out in the presence of 6 mol/l urea, 28 S RNA was degraded to the same high molecular mass fraction, but the smaller cleavage products were separated more distinctly and yielded 6—7 bands (Fig. 3-IV). The results were the same with 28 S RNA from mouse embryo cells.

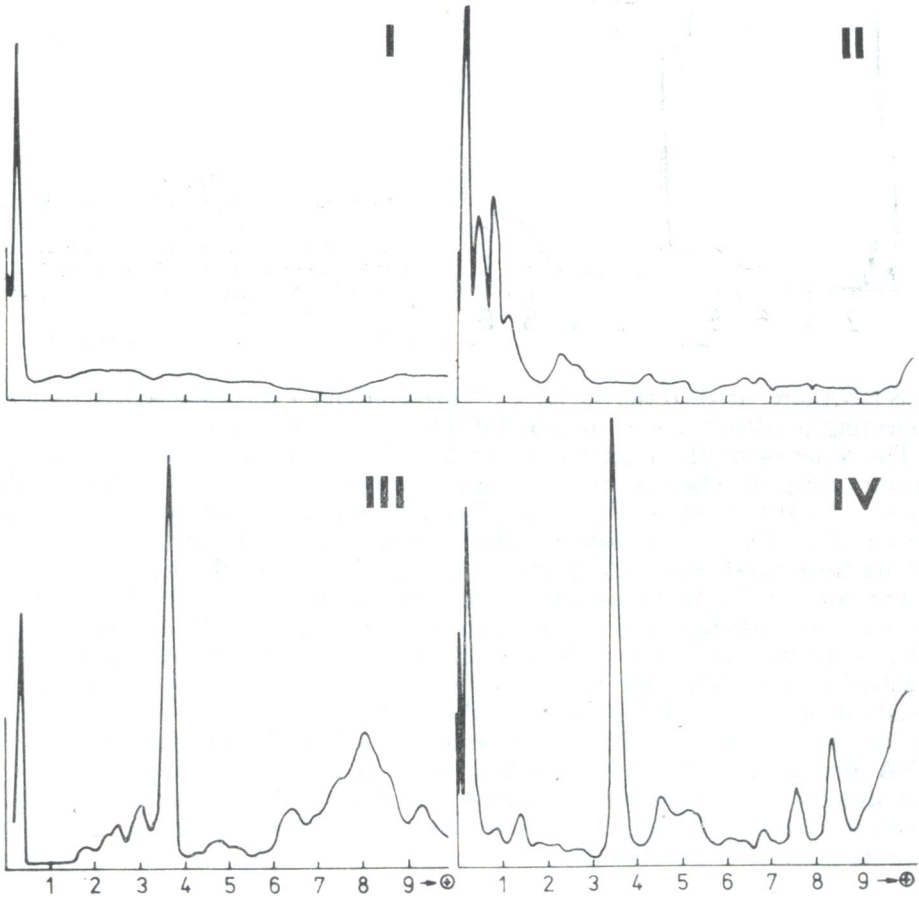

Fig. 3.

Cleavage of 28 S RNA from Ehrlich ascitic cells with NDV
The PAG electrophoretic profiles. 100 μg of viral protein alone incubated in TNE-urea buffer (I), 28 S RNA incubated in TNE-urea buffer (II), 28 S RNA incubated with NDV in TNE buffer (III), 28 S RNA incubated with NDV in TNE-urea buffer (IV).
Abscissa: migration distance in cm

The molecular mass (Mr) of the high-molecular cleavage product shown in Fig. 3-III was estimated by sedimentation analysis and found to be $1.700 \pm 0.085 \times 10^5$. This value correlated well with that determined by PAG electrophoresis ($Mr = 1.6 \times 10^5$). As molecular mass markers in electrophoresis the 23 S and 16 S RNA from *Streptomyces aureofaciens* ($Mr = 1.07 \times 10^6$ and 0.56×10^6) and 18 S RNA from Ehrlich ascitic cells ($Mr = 0.7 \times 10^6$) were used.

18 S RNA after incubation with TNE-urea buffer migrated in electrophoresis about 1 cm from the top of the gel (Fig. 4-I). In the reaction with NDV the

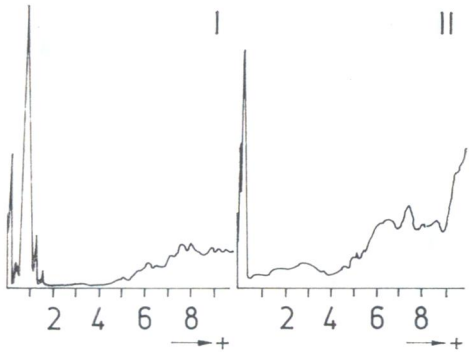

Fig. 4.
Cleavage of 18 S RNA from Ehrlich ascitic cells with NDV
The PAG electrophoretic profiles. 18 S RNA incubated in TNE-urea buffer (I), 18 S RNA incubated with NDV in TNE-urea buffer (II).
Abscissa: migration distance in cm

18 S RNA did not yield the Mr 1.7×10^5 product and it was cleaved to a material migrating markedly faster in broad diffuse bands (Fig. 4-II).

The kinetics of the degradation of 28 S RNA in the presence of urea is shown in Fig. 5. The first two fractions gradually disappear and the fraction Mr $= 1.7 \times 10^5$ increases (Fig. 5-II). The latter remains resistant to the enzyme action after 5 hr of incubation (Fig. 5-III), or even, following addition of 100 μg fresh viral protein 3 hr after the beginning of incubation.

The aim of further experiments was to estimate the proportion of the Mr 1.7×10^5 cleavage product in the input substrate. 28 S RNA labelled with ^{3}H-uridine was used as a substrate in the enzymic reaction (Fig. 6). After incubation with NDV for 4 hr at 37 °C almost all RNA was degraded. The resistant Mr $= 1.7 \times 10^5$ product in PAG migrated in the fraction No. 15; it represented about 10% of the input substrate (Table 1). The majority of 28 S RNA incubated in the absence of the virus did not migrate in the gel, and it was detected in the first two fractions in PAG (Fig. 6). Small portions of radioactivity were detected in the region of 18 S (fraction No. 4) and in fast migrating fraction (fraction No. 26).

Discussion

rRNAs from Ehrlich ascitic or mouse embryo cells are degraded by the NDV-associated endonuclease at 30—40 °C, (i.e. under conditions similar to those in vivo), yielding preferentially high molecular weight products. At

Table 1. Cleavage of 28 S RNA to product of Mr $= 1.7 \times 10^5$

Exp. No.	Radioactivity (c.p.m.)		Per cent of radioactivity in the product
	28 S RNA	product	
1	209,760	22,324	10.9
2	254,110	23,040	9.1
3	167,560	16,635	9.9

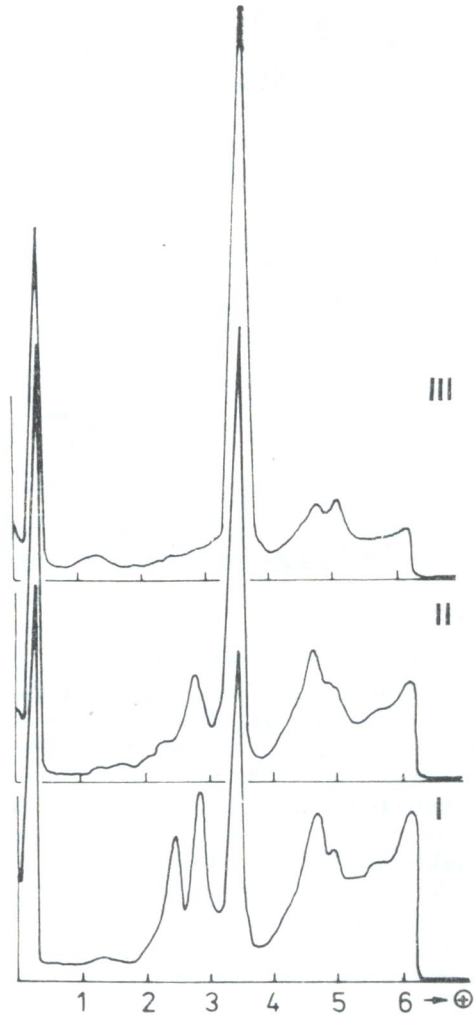

Fig. 5.
Time course of the cleavage of 28 S RNA from Ehrlich ascitic cells with NDV at 37 °C.
The PAG electrophoretic profiles: 28 S RNA incubated in TNE-urea buffer for 15 (I), 60 (II) and 300 (III) min.
Abscissa: migration distance in cm.

higher temperatures (up to 60 °C), the degradation proceeds to oligonucleotides (Rosenbergová and Pristašová, 1972). The enzyme associated with NDV displays a marked difference in the cleavage of 28 S and 18 S RNAs; the former species is degraded preferentially to a polynucleotide of Mr $= 1.7 \times 10^5$ which can be detected already after 15 min incubation at 37 °C and accumulates during the incubation period. This polynucleotide fraction is resistant to a prolonged treatment with the enzyme. Under similar conditions, the 18 S RNA is degraded to smaller products which migrate in electrophoresis in diffuse bands.

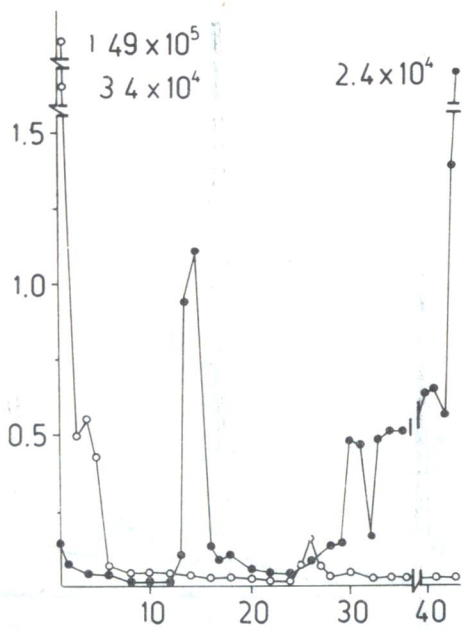

Fig. 6.
Cleavage of 28 S RNA from L cells with NDV
Electrophoretic profile in PAG; ⊙——⊙ radioactivity of 28 S RNA, ●——● radioactivity of 28 S RNA incubated in TNE-urea buffer pH 7.4 with NDV for 4 hr.
Abscissa: fraction number
Ordinate: radioactivity in c.p.m. $\times 10^{-4}$.

On heating in the presence of urea, the rRNAs undergo a partial dissociation which is demonstrated in a marked change of sedimentation constants (Fig. 2). The substantial part of 28 S RNA sediments under these conditions in sucrose gradient at the position of 18 S RNA. This RNA contains segments resistant to the action of the above endonuclease and the 1.7×10^5 Mr material can be detected as the cleavage product (results not shown).

The radioactivity estimation of cleavage products of 28 S RNA under the conditions of the total degradation of the substrate reveals that about 10% of the total cleavage products occurs in the fraction of Mr $= 1.7 \times 10^5$. Since the molecular weight of this fraction represents about one tenth of that of the 28 S RNA, we can assume that there is approximately one resistant segment in one molecule of the 28 S RNA.

The analysis of the products of the enzymic cleavage was performed by electrophoresis in PAG containing 6 mol/l urea to reduce the aggregation of cleavage products and to reveal hidden breaks in rRNAs (Pene *et al.*, 1968; Weinberg and Penman, 1968; Fujisawa *et al.*, 1973) which could mimic the degrading action of the endonuclease under study. Heating of rRNAs in the presence of 6 mol/l urea led to their partial dissociation; the electrophoresis generally displayed a product migrating in a distinct peak. However, this fraction did not interfere with the products of enzymic cleavage, because of the difference in size and its degradation by the enzyme to smaller molecules.

Though no elucidation of the mechanism of action of the NDV-associated endonuclease of rRNAs was attempted, we assume that the primary structure of rRNAs is not the only cause of the resistance of their relatively long segments

to the enzyme. The secondary and tertiary structure of 28 S RNA may also play a role in this phenomenon. Our results obtained in the cleavage in the presence of 6 mol/l urea are not contradictory to this assumption, because RNAs are not completely denatured under these conditions (Reijnders *et al.*, 1973).

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