

## CHARACTERISTICS OF THE RIBONUCLEOPROTEIN OF INFLUENZA VIRUS A

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*Summary.* — Ribonucleoproteins (RNP) of influenza viruses A/Singapore/1/57, A/Victoria/35/72 and those isolated in the course of passaging in persistent infection systems (influenza virus — diploid human lung cells) were subjected to sedimentation analysis. In viruses of different antigenic structure the 3 RNP fragments had the same sedimentation coefficients (63, 53 and 42 S, respectively). The ratios of RNP fragment concentrations had an individual character and were in relation with the antigenic differences between the strains studied.

*Key words:* influenza virus; ribonucleoprotein; sedimentation analysis

### Introduction

The fragmented nature of influenza virus RNP has been generally accepted but it remains obscure whether it is strain-specific. A comparison of data related to this problem is difficult to due the various methods of virion disintegration and the various approaches in determining the RNP structure and properties used.

Gitelman *et al.* (1973) and Zhilinskaya *et al.* (1974) showed that RNPs of various influenza viruses A clearly differ from one another in particular by the concentration ratios of the individual fragments. In the present work we continued to study this problem at maximal standardization of methods of isolation and characterization of the viral RNP. With this aim

1. RNP was isolated from "standard" suspensions of virions based on size (100—130 nm) and sedimentation properties ( $s_{20,w}^0 = 760$  S;  $\rho_{\text{sucrose}} = 1.21$  g/cm<sup>3</sup>) Zhukova *et al.* 1975);

2. virions were disintegrated by the non-ionic detergent NP-40 which yields RNP practically free from envelope material (Pons, 1971) and containing almost all RNA (Schulze 1970); and

3. RNP fragments were investigated immediately after virion disintegration without their previous isolation by differential centrifugation.

The individual character of the heterogeneity of RNP was investigated on two reference strains of influenza virus A — A/Singapore/1/57 (H2N2) and

A/Victoria/35/72 (H3N2) — and on viruses isolated from systems of persistent infection in diploid human lung (DHL) cell cultures. The method of inducing persistent influenza infection was described (Medvedeva, 1975; Golubev *et al.*, 1976, p 155). The persisting viruses differed in their antigenic structure and biological properties from the original viruses used for inducing the persistent infection. Two such persisting viruses, isolated from different systems, were used in the present work. Virus I was identified as H2N2 (“Singapore-like”) while the original virus was H3N2 (virus A/Victoria/35/72). Virus II was “Victoria-like” (H3N2) while the original virus was H2N2 (Medvedeva and Golubev 1977).

### Materials and Methods

*Viruses.* Reference strains A/Singapore/1/57 (H2N2) and A/Victoria/35/72 (H3N2) and persistent variants I and II were propagated by inoculating chick embryos into the allantoic cavities with doses of  $10^2$ – $10^3$  EID<sub>50</sub>/0.1 ml. Virus was purified and concentrated from the allantoic fluids whose initial infectivity and haemagglutinin titres were 7–8 log EID<sub>50</sub>/0.1 ml and 100–1000, respectively, as follows:

1. Differential centrifugation at  $10000 \times g$  for 15 min and  $40000 \times g$  for 60 min at 5 °C in a TSVR-I (U.S.S.R.) centrifuge; the pellet was resuspended in STE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA);

2. isopycnic centrifugation in a 10–66 % sucrose gradient in STE buffer (25000 rev/min for 8 hr at 5 °C in the SW 25.1 rotor of a Beckman L4 centrifuge);

3. rate zonal centrifugation in a 10–40 % sucrose density gradient in STE buffer (25000 rev/min for 20 min at 5 °C in the SW 25.1 rotor of a Beckman L4 centrifuge). Finally, the virus was suspended in STE buffer. The suspensions of standard virions with a sedimentation coefficient of 760 S and a buoyant density in sucrose of 1.21 g/cm<sup>3</sup> had haemagglutinin titres of  $10^6$  at a protein concentration of 10–12 mg/ml.

The virions were disintegrated with the non-ionic detergent NP-40 and urea (in final concentrations of 1 % and 0.5 M respectively) at 20 °C immediately before being subjected to analytical centrifugation.

The latter was carried out in a Beckman model E centrifuge with a standard 30–mm cell of

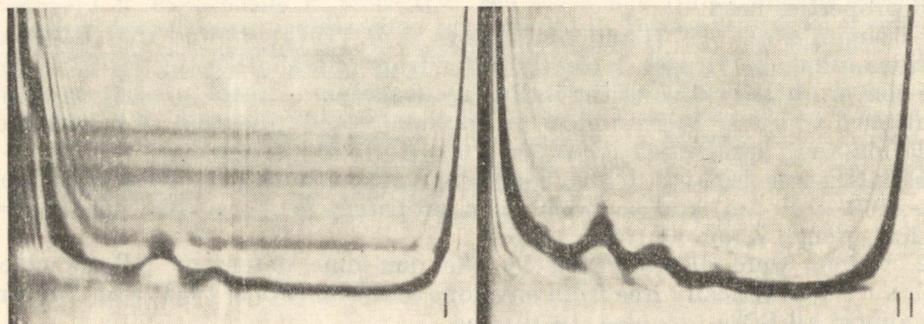


Fig. 1.

Sedimentation pattern of A/Singapore/1/57 (I) and A/Victoria/35/72 (II) viruses treated with 1% NP-40 and 0.5 M urea; 28000 rev/min; 20 °C; AnD, model E Beckman analytical centrifuge

**Table 1. Characteristics of RNP of reference and "persistent" influenza viruses as revealed by sedimentation analysis**

Virus	No. of experiments	Relative concentration of RNP fragments in % ( $M \pm m$ )		
		63 S	53 S	42 S
A/Singapore/1/57	5	66.3 $\pm$ 1.8	24.7 $\pm$ 1.3	9.0 $\pm$ 0.8
Virus I	4	70.8 $\pm$ 3.7	23.6 $\pm$ 1.0	5.6 $\pm$ 0.3
A/Victoria/35/72	6	54.0 $\pm$ 0.9	38.2 $\pm$ 0.7	7.8 $\pm$ 0.4
Virus II	5	58.2 $\pm$ 2.0	36.0 $\pm$ 0.9	5.8 $\pm$ 0.4

The values in the table represent means  $\pm$  standard deviations calculated by Student's *t*-test. In each experiment, 7–8 schlieren pictures were measured.

the An-E rotor at 28000 rev/min and at 20 °C, using Schlieren optics. The sedimentation coefficients were calculated for infinite dilution ( $s_{0,20,w}^0$ ).

The relative concentrations of RNP fragments in the test suspensions were determined by measuring the surface areas of the Schlieren peaks, taking into account radial dilution and the Schlieren-element angle.

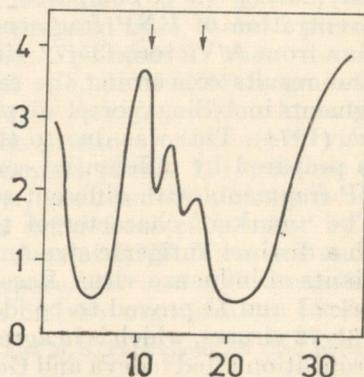
*Isolation of individual RNP fragments.* Suspensions of disintegrated virions were layered on 15–30% linear sucrose gradients in STE buffer and centrifuged for 16 hr at 19000 rev/min and at 5 °C in the SW 25.1 rotor of an L4 centrifuge. Fractions were collected by an LKB fraction collector with continuous recording of the optical density at 260 nm.

*Serology.* Each RNP fragment was identified in complement-fixation (CF) tests with anti-S and anti-V sera. Anti-S sera were prepared essentially as described by Závadová *et al.* (1967) and anti-V sera according to Lief and Henle (1959).

### Results

Analytical centrifugation of NP-40-disintegrated virions revealed three RNP fragments in all the viruses tested (Fig. 1). The sedimentation coefficients  $s_{0,20,w}^0$  of the corresponding RNP fragments were the same in all the viruses tested and were 63 S for fragment I, 53 S for fragment II and 42 S for fragment III.

The individual RNP fragments were prepared from the disintegrated viruses by centrifugation in linear sucrose density gradients. The sedimenta-

**Fig. 2.**

Sedimentation pattern of A/Singapore/1/57 virus RNP in sucrose density gradient. Purified virus was treated with 1% NP-40 and 0.5 M urea and centrifuged in a 15–30% sucrose gradient for 16 hr at 19000 rev/min and at 5 °C in the SW 25.1 rotor of an L-4 centrifuge. Arrows indicate position of 60 S and 40 S ribosomal subunits centrifuged in a separate tube. Abscissa: fraction number, ordinate:  $OD_{260}$  in arbitrary units

Fig. 1 shows that, in spite of the same sedimentation coefficient values, the peak surface areas for A/Singapore/1/57 and A/Victoria/35/72 viruses differed from one another. Consequently also the relative concentrations of the individual RNP fragments in these viruses were different (Table 1).

tion patterns (records of optical density at 260 nm) were the same with all the viruses tested. The result obtained with A/Singapore/1/57 virus is presented as an example in Fig. 2. Pooled fractions 10–11 (I), 13–14 (II) and 16–17 (III) corresponding to the individual RNP fragments were freed from sucrose by dialysis against STE buffer and examined in CF tests. All fragments of each of the viruses tested reacted only with anti-S sera. No reactions were obtained with anti-V sera.

Electrophoresis showed each RNP fragment to contain a major polypeptide with a molecular weight of 57000 and a minor polypeptide with a molecular weight of 80000–100000.

### Discussion

Sedimentation analysis of RNP of two reference and two "persistent" strains of influenza virus A revealed three classes of fragments. The sedimentation coefficients of the corresponding RNP fragments were the same in all these viruses and were 63, 53 and 42 S, respectively. These results are in accordance with previous data on RNP fragments of A/PR8 (Paucker *et al.*, 1959) and A/Singapore (Hjertén *et al.*, 1970; Zhilinskaya *et al.*, 1974) viruses. However, in preparations of RNP of fowl plague (Schäfer and Zillig, 1954) and A/Hong Kong (Zhilinskaya *et al.*, 1974), a fourth minor 20–25 S component was demonstrated. According to our results (Zhukova *et al.*, 1975b), this component was the result of partial degradation of the RNP during its concentration by differential centrifugation.

A comparison of the relative concentrations of these RNP fragments in the viruses tested (see Table 1) showed that the content of the largest fragments (63 S) in the RNP of A/Singapore/1/57 virus was significantly higher than in the RNP of A/Victoria/35/72 virus ( $66.3 \pm 1.8\%$  compared with  $54.0 \pm 0.9\%$  respectively). The opposite was true for the 53 S fragment ( $24.7 \pm 1.3\%$  compared with  $38.2 \pm 0.8\%$ ). Based on the relative concentration of RNP fragments A/Singapore/1/57 virus thus significantly differs from A/Victoria/35/72 virus.

Our results concerning the relative concentration of the individual RNP fragments in A/Singapore/1/57 virus differ from those obtained by Zhilinskaya *et al.* (1974). This was due to the fact that in previous investigations RNP was prepared by differential centrifugation which alters the actual ratio of RNP fragments with different sedimentation coefficients.

The "marker" character of the sedimentation profile of RNP of viruses with a distinct antigenic structure was confirmed in studies on the persisting variants of influenza virus. Based on the sedimentation profile of their RNP, viruses I and II proved to be identical with A/Singapore/1/57 and A/Victoria/35/72 viruses, which is in agreement with the results of their immunological classification (Medvedeva and Golubev, 1977).

The results of sedimentation analysis make it possible to evaluate the geometric parameters of the individual RNP fragments. Considering that RNP fragments hydrodynamically represent solid cylindrical particles with evenly distributed mass along the long axis, Perren's equation (Johnson *et al.*, 1963) can be used in relating the sedimentation coefficients with the parameters of such particles:

$$s_{20,w}^0 = \frac{1 - \bar{v}\rho}{\bar{v}\eta} \times \frac{2}{9} a^2 \ln \frac{2b}{a}$$

where  $\bar{v}$  = partial specific volume of the particle;  $\rho$  and  $\eta$  = density and viscosity, respectively, of the solvent at 20 °C;  $a$ ,  $b$  = the short and long axis of the cylinder, respectively.

Taking into account that RNP consist of 90% protein and 10% RNA (Schulze *et al.*, 1970), based on the partial volumes of protein and RNA — 0.75 and 0.55 cm<sup>3</sup>/g respectively (Atabekov, 1966), we calculated the partial specific volume of RNP as 0.73 cm<sup>3</sup>/g. Density and viscosity of the solvent were 1.0 g/cm<sup>3</sup> and 25 n × m<sup>2</sup>, respectively.

Taking the value of the axial ratio  $2b/a$  for the large fragment to be 20 (Compans *et al.*, 1972), the thickness of the fragment can be calculated from the known sedimentation coefficient. It was equal to 10, which is in accordance of electron microscopy data (Compans *et al.*, 1972). Assuming the same thickness for all RNP fragments, the equation can be used for calculation of their length. This was approx. 100 nm for fragment I (63 S), 60 nm for fragment II (53 S) and 40 nm for fragment III (42 S), which is in accordance with direct electron microscopic measurements (Compans *et al.*, 1972).

If intravirion RNP consists of the same number of fragments of each class, then the relative concentrations of the fragments should be proportional to their length, i. e. the first, second and third fragments should represent 50, 30 and 20 per cent of the total mass, respectively.

Comparing the actual concentration ratios (Table 1) with the hypothetical model of an equal number of the different RNP fragments in the virion, we can calculate the minimal number of pieces of each class of RNP fragments in virions of different strains. This calculation showed that in A/Singapore/1/57 virus and the antigenically similar virus I, the first (63 S), second (53 S) and the third (42 S) class of fragments is represented by 5, 3 and 2 pieces of RNP, respectively. For A/Victoria/35/72 virus and virus II these numbers were 3, 3 and 1, respectively. Thus the total number of RNP fragments in influenza virus A is 7–10. This is in accordance with the present knowledge about the number of RNP fragments in the genomes of influenza viruses as revealed by polyacrylamide gel electrophoresis (Pons, 1976; Palese and Schulman, 1976; Hay *et al.*, 1977). Moreover, the results obtained indicate that A/Singapore/1/57 and A/Victoria/35/72 differ from each other by the number of fragments in each sedimentation class of RNP. Viruses I and II, similar in their antigenic profile to A/Singapore/1/57 and A/Victoria/35/72 viruses, respectively, resembled them also in the organization of RNP.

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