

Structural Studies of the Ribonucleoprotein G-Antigen of Influenza Virus

M. J. SPARKS LEVITCH and EDWARD A. ECKERT

Department of Epidemiology, School of Public Health, University of Michigan,
Ann Arbor, Michigan 48104, U.S.A.

*This work is dedicated to our friend and colleague Professor WERNER SCHÄFER
on the occasion of his 60th birthday*

(Z. Naturforsch. 27 b, 246—251 [1972]; received December 31, 1971)

The physical and serological states of ribonucleoprotein antigen, released by dissociation of influenza virus, have been characterized. While the simplest antigenic derivative has a sedimentation constant of 4s, more complex antigenic forms, 19s, 38s, and 60s have been identified and isolated. The physical dimensions of isolated g-antigen are dependent on the form of pretreatment of virus concentrate and on the method of virus disruption. The properties of several isolated substructures vary, depending on interaction of the protein with host antigen and nucleic acid.

The results suggest that influenza virus ribonucleoprotein associates readily with host antigens and that this interaction plays a significant role in initiating the process of budding which leads to release of mature virus.

Purified influenza virus, on disruption of the virion by chemical reagents, releases ribonucleoprotein as serologically active g-antigen^{1,2}. While antigenic specificity remains essentially constant, the physical states of the antigen recovered in several laboratories have varied markedly. It was apparent that the nature of disruptive procedures, and perhaps the strain of virus and purification methods used, determined the conformation of released ribonucleoprotein macromolecular structures.

In the present study, a single method of virus disruption, ether splitting, has been applied to strain Ao/PR8/34 influenza virus concentrates which have been treated with enzymes prior to ether disruption. In this way, three distinct kinds of substructures, all containing viral ribonucleoprotein antigen, have been identified and isolated reproducibly. Serological and physical characteristics of the several forms of the antigen have been studied.

Materials and Methods

Virus

Influenza virus, strain Ao/PR8/34, was inoculated into the allantoic cavity of 11 day old embryonated hen's eggs; each egg received 100 EID₅₀. Inoculated eggs were incubated for 72 hours at 35 °C prior to collection of allantoic fluid.

Requests for reprints should be sent to Prof. Dr. E. A. ECKERT, Dept. of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104, U.S.A.

Virus was separated from allantoic fluid and concentrated 10 fold by adsorption to and elution from chicken erythrocytes. Further purification was accomplished by treatment with barium sulfate according to the method of DRESHER et al.³. Virus was pelleted from barium sulfate eluate by centrifugation at 56,000 g for 1 hour and resuspended in phosphate buffered saline (PBS), 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4 with 0.08 percent sodium azide as preservative. Virus was banded in a 20–70 percent sucrose gradient at 100,000 g for 2 hours in an SW-29 rotor (Spinco). The band was collected as a 1,500 x concentrate and dialyzed against PBS before use.

Antisera

Ribonucleoprotein antisera were prepared in mice using a Type A equine strain of virus, which presumably has no viral antigens, other than g-antigen, capable of cross-reacting with PR8 strain of influenza virus. Mice were inoculated intranasally with 10⁻¹, 10⁻², 10⁻³ dilutions (0.05 ml/mouse) of a lung suspension from mice infected with a mouse adapted equine strain, A/Eq-1/Det/3/64. Four weeks later survivors were re-inoculated with a 10⁻¹ dilution. Two months later mice were injected intraperitoneally with 0.5 ml of a 10⁻¹ dilution, and reinjected with the same dose after one week. Mice were then bled one week later. Sera were inactivated at 56° for 30 min and stored at -20 °C. These sera did not inhibit hemagglutination by the PR8 strain of influenza virus, had no detectable complement fixing antibody against chorioallantoic membrane antigen and had a complement fixation titer of 128 with ribonucleoprotein antigen.

Antisera to normal host (egg) material were prepared by inoculation of rabbits with 0.5 ml of a 10 percent (V/V) suspension of homogenized chorioallantoic membranes clarified by low speed centrifugation. Biweekly intravenous inoculations were given over a

four week period and the animals bled one week after the last inoculation. Sera were inactivated and stored at -20°C .

Serological tests

Viral hemagglutination was measured by the micro test of Sever. Serological titers for virus ribonucleoprotein antigen and normal host antigens were determined by micro-complement fixation ⁴.

Gradient centrifugation

Rate zonal sedimentation studies were carried out in 4 ml sucrose gradients (5–20 percent in PBS). Samples were centrifuged at 100,000 *g* in a Spinco Model L centrifuge at 4°C , using an SW-39 rotor. Fractions (0.5 ml) were collected from the top of the gradient with a one ml tuberculin syringe. Reference proteins used as markers of sedimentation rates were: IgM anti-typhoid O antibody (19s) and Fraction V, bovine albumin (4.6s).

Density studies utilized self-forming gradients of cesium chloride in PBS. Samples contained in 3 ml volumes were mixed with 1.2 ml of a saturated aqueous solution of CsCl and centrifuged at 100,000 *g* for 66 hours at 4°C . Fractions were collected from the top of the gradient and density measurements were made by weighing aliquots contained in 0.1 ml capillary pipettes. It was necessary to remove CsCl by dialysis prior to complement fixation tests.

Ether Disruption

Aliquots of virus concentrate were vigorously shaken with a half volume of ether for 2 hours at 37°C . The phases were separated by low speed centrifugation and the aqueous phase removed by capillary pipette. Nitrogen was bubbled through the aqueous phase to remove ether and viral hemagglutinin removed by three adsorptions with 30 percent chicken erythrocytes.

Results

Preparation of 60s, 38s, and 19s ribonucleoprotein antigens

Three different types of virus preparations, the original virus concentrate and two modified by enzymic digestion, were split by shaking with ether and the sedimentation characteristics of the products compared. "Trypsinized virus" was prepared by treatment of virus concentrate with crystalline trypsin in a final concentration of 0.2 percent for 30 min at 37°C , banding on a 20–70% sucrose gradient and dialysis against PBS to remove sucrose. Aliquots of trypsinized virus were additionally exposed to ribonuclease, 50–1000 μg m per ml of virus concentrate, for 2 hours at room temperature

to yield "ribonuclease treated trypsinized virus". Enzyme digested preparations and the original virus concentrate were then subjected to ether splitting and adsorbed with chicken erythrocytes to remove residual hemagglutinin. The samples were layered over 5–20 percent sucrose gradients and centrifuged at 100,000 *g* for 2–3½ hours. Fractions were collected and tested for ribonucleoprotein and host antigen by complement fixation tests. IgM, with a sedimentation constant of 19s, was used as a marker permitting calculation of sedimentation constants of the ribonucleoprotein antigens.

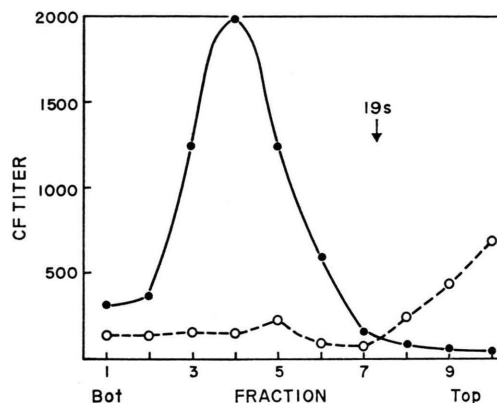


Fig. 1. Rate zonal centrifugation of ether-split influenza virus concentrate. Solid circles (●—●) refer to complement fixation titers of ribonucleoprotein antigen and open circles (○—○) to host antigen titers. Centrifugation was in a 5–20 percent sucrose gradient at 100,000 *g* for 2 hours.

As shown in Fig. 1, ribonucleoprotein antigen, obtained by ether-splitting of untreated virus concentrate, was detected as a relatively sharp peak in the lower half of the tube providing an estimated sedimentation constant of 60s. Some host antigen was present in all fractions but most was found as slowly sedimenting components.

Qualitatively similar results (Fig. 2) were obtained with ether-split trypsinized virus, but the peak of ribonucleoprotein antigen was located closer to the 19s marker and the sedimentation constant was estimated at 38s. Most host antigen was detected in the top fractions of the gradient.

Application of the procedure to ether-split ribonuclease treated trypsinized virus gave a similar single peak of ribonucleoprotein antigen, but in this case, the sedimentation constant was 19s, identical to the IgM marker (Fig. 3). Recovery of antigen was considerably reduced as compared to the pre-

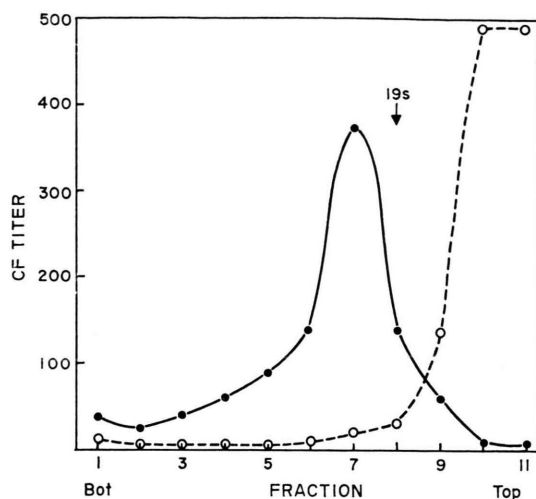


Fig. 2. Rate zonal centrifugation of ether-split "trypsinized influenza virus concentrate". See Fig. 1 for details.

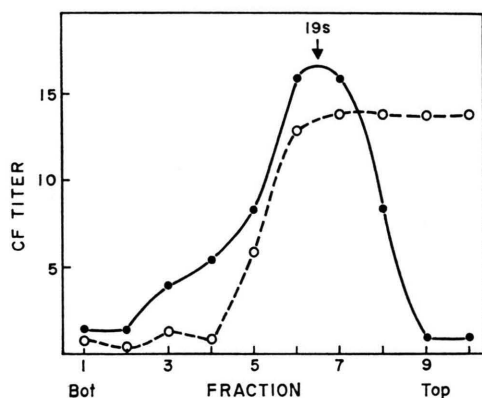


Fig. 3. Rate zonal centrifugation of ether-split ribonuclease treated trypsinized influenza virus concentrate. Details are as in Fig. 1 except that centrifugation was for 3½ hours.

vious procedures. While host antigen again sedimented slowly, it was spread further down the tube, overlapping the peak of ribonucleoprotein.

Thus, pretreatment of virus concentration with enzymes alters the structure of the virion so that ribonucleoprotein antigen components differing in their sedimentation rates can be detected following ether-splitting. Further, by rate zonal centrifugation, it is possible to isolate the 19s, 38s and 60s forms of the antigen in serologically active states.

Comparing the above results with dissociation products obtained by exposure to detergents, some differences are noteworthy. Sodium dodecyl sulfate (SDS), at a concentration of 0.1 percent, reduced trypsinized virus to 38s substructures which are distinct from the 19s hemagglutinin. However, on

reduction of SDS concentration by dialysis, the ribonucleoprotein antigen titer dropped markedly. The dissociation products obtained with sodium deoxycholate (DOC) were found to be dependent on the concentration of detergent. Over the range of 1–8 percent, sedimentation constants decreased from about 40s to 15s as DOC concentration was increased. Following dialysis to minimize residual DOC, ribonucleoprotein as well as hemagglutinin was removed by adsorption with erythrocytes (Table 1). This finding indicates that DOC disso-

DOC concentration [percent]	Adsorption	HA titer	Ribo-nucleoprotein titer
1	pre	512	16
	post	< 2	< 2
4	pre	128	16
	post	< 2	< 2

Table 1. Adsorption of DOC Treated Virus with Chicken Erythrocytes.

ciation fails to completely separate both viral components or that association occurs during removal of the DOC. The results emphasize certain advantages of the ether-splitting procedure, uniformity of product, freedom from bound detergents and avoidance of clumping on removal of dissociating reagents.

Dissociation of isolated ribonucleoprotein antigen preparations by detergents

Isolated ribonucleoprotein antigen preparations were treated with detergents in an attempt to identify the basic subunits. Aliquots of 60s ribonucleoprotein antigen, prepared from ether-split virus concentrates, were exposed to 0.1% SDS for two hours at room temperature, then immediately centrifuged on a 5–20 percent sucrose gradient containing 0.1 percent SDS. The sharp drop in antigenic titer, e.g., from 2048 to 32, precluded useful serological tests, but the fractions were analyzed by UV adsorption at 280 nm. Fig. 4 compares the sedimentation pattern of SDS treated ribonucleoprotein antigen with the original sample. The results demonstrate that 60s ribonucleoprotein antigen was dissociated by SDS into 4s subunits. While some of the UV adsorption may be due to host antigen, other experiments indicate that its contribution is minor.

On similar treatment of a 38s ribonucleoprotein preparation with 0.1 percent SDS, antigenic titers

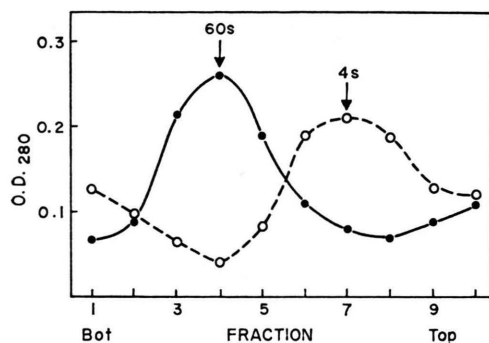


Fig. 4. Rate zonal centrifugations of 60s ribonucleoprotein antigen pre- and post-treatment with 0.1% SDS. Solid circles (●—●) refer to 60s antigen centrifuged in a 5–20 percent sucrose gradient at 100,000 g for 2 hours and open circles (○—○) to SDS treated 60s antigen centrifuged at 100,000 g for 16 hours. Distribution of protein was measured by adsorption at 280 nm.

dropped 5-fold so that residual activity was sufficient to permit serological identification of ribonucleoprotein in the sucrose gradient fractions. The sedimentation profile was broad with a maximum at 7s and a secondary peak at 4s. Reduction of SDS concentration by dialysis and centrifugation in a sucrose gradient without SDS resulted in a complexing of antigen to a more rapidly sedimenting polydisperse state. In an attempt to limit inactivation by SDS and association, 38s ribonucleoprotein antigen was treated with 0.1 percent SDS for 2 hours, then layered over a sucrose gradient containing 1 M guanidine as a dispersing agent instead of SDS. While the sedimentation profile (Fig. 5) shows that a major portion of the antigen is at the bottom of

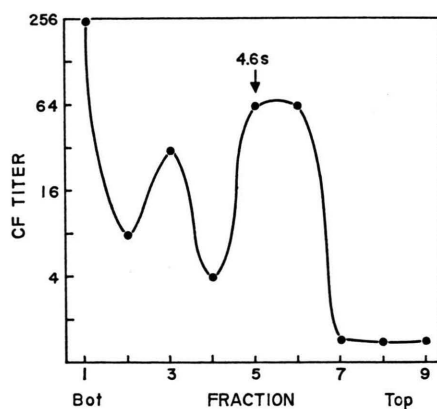


Fig. 5. Rate zonal centrifugation of 38s ribonucleoprotein antigen after dissociation with 0.1 percent SDS. Centrifugation was in a 5–20 percent sucrose gradient containing 1 M guanidine at 100,000 g for 16 hours. Titers refer to ribonucleoprotein antigen complement fixation titers.

the tube in an aggregated form, distinct peaks of serological activity were located at the 4s and 7s positions.

Dissociation of isolated ribonucleoprotein antigen preparations by ribonuclease

To determine whether virus ribonucleic acid stabilized configurations, the several macromolecular forms of ribonucleoprotein antigen were treated with ribonuclease. The 38s antigen was held for 2 hours at room temperature in the presence of ribonuclease, 50–100 μ gm per ml, then centrifuged over a 5–20 percent sucrose gradient for 16 hours at 100,000 g . As shown in Fig. 6, serological activity

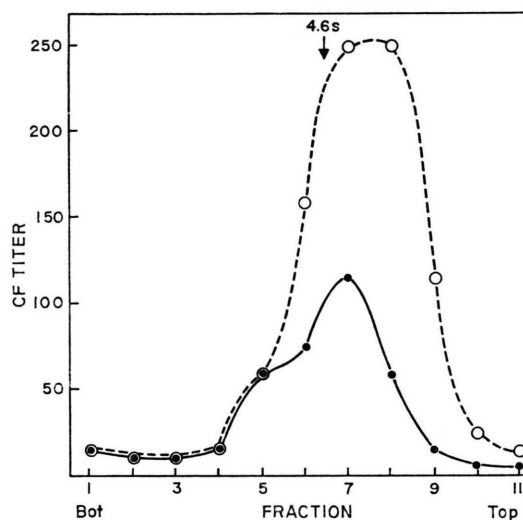


Fig. 6. Rate zonal centrifugation of ribonuclease treated 38s ribonucleoprotein antigen of influenza virus. Solid circles (●—●) refer to ribonucleoprotein antigen titers and open circles (○—○) to host antigen titers. Centrifugation was in a 5–20 percent sucrose gradient at 100,000 g for 16 hours.

peaked at a sedimentation constant of 4s, while there was a shoulder in the region of 7s. Unlike the product obtained by SDS dissociation, which is held in the 4s state by SDS or guanidine, the 4s/7s entities produced by ribonuclease digestion appear to be in a stable state. Thus, overnight dialysis of ribonuclease digested antigen prior to centrifugation did not alter the sedimentation profile. Ribonuclease treatment resulted in a 4–5 fold drop in complement fixation titer and immunodiffusion patterns, readily attained with large size antigen, could not be detected with 4s/7s antigen at the concentrations available. Host antigen was also localized at the 4s position.

In contrast to 38s antigen, neither the 60s nor 19s forms of ribonucleoprotein antigen were dissociated by ribonuclease treatment, the sedimentation profiles remaining identical. There is evidence that host antigen plays a role in this stabilization since when ribonuclease digestion of 60s antigen was carried out in the presence of anti-host serum, there was a significant, although not complete, conversion to the 4s/7s state. If aliquots of antigen were treated with ribonuclease with and without anti-host serum and analyzed by sucrose gradient centrifugation, the titer at the 4s fraction was only 2^2 in the absence of serum but increased to 2^5 in the presence of serum.

Isopycnic centrifugation

Equilibrium centrifugation in cesium chloride of 38s ribonucleoprotein antigen gave a single peak at 1.29 gm/ml (Fig. 7). When an aliquot was di-

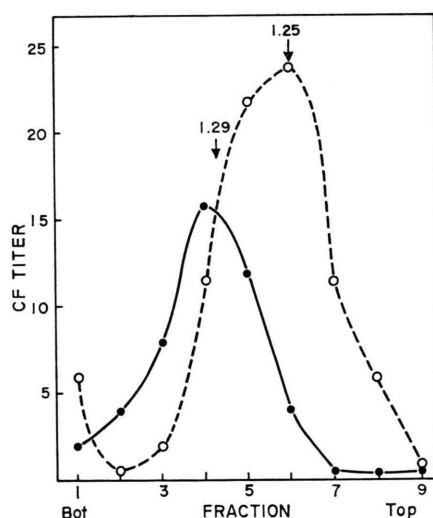


Fig. 7. Equilibrium centrifugation of 38s ribonucleoprotein antigen in cesium chloride at 100,000 g for 66 hours. Solid circles (●—●) refer to ribonucleoprotein antigen titers and open circles (○—○) to host antigen titers.

gested with ribonuclease prior to centrifugation, the distribution was essentially the same. Lack of a shift to a lower density following enzymic digestion is in accord with the observation of KRUG⁵ that protein and nucleic acid are dissociated in cesium chloride. In contrast to ribonucleoprotein antigen, the host antigen titer reached a maximum at 1.25 mg/ml.

Discussion

PONS *et al.*⁶ detected a 4s protein subunit derived from radioactively labelled influenza virus ribonucleoprotein and an estimated molecular weight of 60,000 has been determined for the polypeptide⁷. The results of the present study are in essential agreement with the above and, in addition, demonstrate that the basic structural unit is serologically active. Nevertheless, reduction of ribonucleoprotein to 4s protein subunits by ribonuclease does lead to a 4–5 fold decrease in complement fixation titers and loss or marked decrease of the capacity to form precipitin lines on immunodiffusion. Thus, viral nucleic acid not only stabilizes the larger substructures of ribonucleoprotein antigen but also maintains conformations consistent with a high level of serological activity.

While 4s subunits obtained by SDS dissociation and ribonuclease digestion have similar dimensions, the former associate on removal of unbound SDS by dialysis, while the latter are stable as 4s units or association is limited to some 7s material. The reassociation of SDS products indicate that either SDS alters the protein, perhaps due to bound SDS, or liberates nucleic acid products which recombine with 4s protein subunits on dialysis.

With regard to the larger substructures of ribonucleoprotein antigen which consist of 4s protein subunits bound perhaps to other intra-virion substances, individual investigators have reported release of several different physical states of the antigen. Thus, DUESBERG⁸ isolated by DOC disruption a 50–70s form of ribonucleoprotein which was resistant to ribonuclease and pronase. In contrast, PONS *et al.*⁶ reported that the 38s component released by the non-ionic detergent P-40 was reduced by ribonuclease to 4s subunits and was sensitive to pronase.

In the present investigation, using a single strain of virus and a uniform method of virus disruption, ether-splitting, ribonucleoprotein antigens with sedimentation constants of 19s, 38s, and 60s have been isolated. The nature of the pretreatment of virus concentrates by enzymes determines the state of the dissociation product. The 60s structure, like that of DUESBERG, is resistant to ribonuclease degradation but it has now been shown that antihist serum facilitates partial dissociation by ribonuclease. One explanation for this finding would be that interaction

between host antigen present on the substructure and its specific antibody alters the conformation so that the antigen becomes susceptible to ribonuclease. However, DUESBERG⁸ has demonstrated release of degraded ribonucleic from 50–70s forms by action of ribonuclease alone. The data presented in this report would therefore conform with a 60s structure consisting of 4s subunits stabilized by host components and nucleic acid. Alteration of both stabilizers, respectively by action of specific antibody and enzyme digestion, appears essential to reduce the substructure to subunit. In contrast, 38s substructures are readily dissociated by ribonuclease digestion into 4s subunits, demonstrating that viral nucleic acid plays the dominant role in maintaining that conformation. PONS⁶ has shown that 38s structures can be maintained with polyvinyl sulfate instead of nucleic acid. The 19s antigen is also not dissociated by ribonuclease but in this case it is probable that the viral nucleic acid is degraded during the preparation of "ribonuclease treated trypsinized virus" prior to ether disruption and that the antigen is stabilized by host components.

The evidence presented in this investigation supports the view that structural alteration of the membrane is a major determinant of the state of released ribonucleoprotein antigen or g-antigen. Evidence that trypsin acts on viral membrane is shown by the release of neuraminidase and break-down of hem-

agglutinin⁹. The presence of host antigen in at least some kinds of released ribonucleoprotein antigen preparations suggests that membrane material is associated with it. In the case of viral disruption with DOC, adsorption of ribonucleoprotein to erythrocytes along with hemagglutinin further demonstrates that more complex viral membrane fragments can be bound to ribonucleoprotein antigen. In summary, enzymic alteration in viral membrane structure appears to determine at which loci ribonucleoprotein-membrane complexes will dissociate on ether disruption of virus.

The results are in accord with the hypothesis of COMPANS and CHOPPIN¹⁰ that there is a strong interaction between nucleocapsid and membrane, or even more specifically membrane protein. Additionally, the internal ribonucleoprotein component, at the time of attachment to cell membrane, may actually initiate the process of budding leading to release of mature virions.

This investigation was conducted under the sponsorship of the Commission on Influenza, Armed Forces Epidemiological Board and was supported by the U.S. Army Medical Research and Development Command, Department of the Army, under Research Contract DADA-17-70-C-0050.

The senior author was a recipient of an NIH traineeship (AI 00060-14) and the publication is in partial fulfillment of the requirements for the Ph.D. degree.

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