

Envelope Protein(s) Derived from Influenza Virus

EDWARD A. ECKERT

Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan

Received for publication 17 February 1966

ABSTRACT

ECKERT, EDWARD A. (University of Michigan, Ann Arbor). Envelope protein(s) derived from influenza virus. *J. Bacteriol.* **91**:1907-1910. 1966.—Lipids were extracted from influenza virus, strain PR8, with methanol-chloroform, and the protein residue was dissolved in 67% glacial acetic acid. Hemagglutinating activity and complement-fixing reactivity were markedly reduced or lost during lipid extraction, and then increased after acetic acid treatment and subsequent dialysis. Evidence is presented that the envelope protein(s) responsible for these activities is dissociated in acetic acid and reassociated at neutral pH.

There has been continued interest in the nature of the surface components of influenza virus responsible for specific viral antigenicity and for hemagglutination. The present study is concerned with the action of a protein-dissociating agent, strong acetic acid, on lipid-extracted influenza virus.

In general, the application of dissociating chemical treatments to proteins results in the production of subunits which can subsequently be reassociated. In the case of certain proteins with a specific biochemical or biological activity, that activity may be retained by the subunit or lost and then regained after reassociation (10). Such procedures have been applied to plant viruses, notably tobacco mosaic virus, in which case the reassociated protein subunits exhibit antigenic activity, and a structural conformation similar to that of intact virus. Limited investigations of like nature have been made with animal viruses.

MATERIALS AND METHODS

Virus. Concentrates of influenza virus, strain PR8, were used throughout. Virus from infected embryonated eggs was concentrated by adsorption-elution with the use of chicken red blood cells, followed by two cycles of low- and high-speed ultracentrifugation ($6,300 \times g$ and $26,360 \times g$). The concentrates, suspended in 0.15 M sodium chloride, averaged 90,000 hemagglutinating units per ml.

PR8 virus antisera. Rabbits were inoculated in the footpads with 2-ml samples consisting of equal volumes of Freund's incomplete adjuvant and virus concentrates. After 6 weeks, they received an additional 1 ml of aqueous virus concentrate intravenously, and serum was collected 1 week later.

S-antigen and antiserum. Allantoic fluid of PR8-infected eggs was the source of antigen. After adsorption with chicken erythrocytes, the fluid was con-

centrated fivefold by dialysis against Carbowax and clarified by centrifugation at $26,360 \times g$ for 1 hr. The supernatant fluid was adsorbed alternately with chicken and guinea pig erythrocytes to remove all measurable hemagglutinin, and the centrifugation was repeated. The supernatant fluid was then centrifuged at $80,730 \times g$ for 2 hr, and the pellets, containing s-antigen, were suspended in 0.15 M sodium chloride to give a final concentration factor of 80-fold.

An anti-s-antigen rabbit serum prepared with fowl plague virus (Rostock strain) was obtained through the courtesy of Werner Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, Germany.

Solubilization of virus proteins. Lipids were extracted from PR8 virus with methanol-chloroform as described by Kates et al. (5) for the lipid analysis of influenza virus. The precipitate obtained was suspended in water, and a stream of nitrogen was bubbled through to remove organic solvents. On addition of glacial acetic acid to a concentration of 67%, the virus suspension, which was turbid, cleared. On low-speed centrifugation, a small sediment formed, and the supernatant fluid was decanted and dialyzed successively against 0.1 M acetic acid, 0.1 M sodium acetate buffer (pH 4.5), and buffered saline (BS; 0.02 M phosphate buffer, pH 7.4, 0.15 M NaCl). During the last dialysis, a precipitate developed which was removed by centrifugation at $12,800 \times g$ for 10 min. The supernatant fluid had a slight bluish turbidity when diluted to the original volume of the virus concentrate.

Hemagglutination (HA) and hemagglutination inhibition (HI) tests. Standard procedures were followed for the measurement of HA and HI titers. Plastic trays were used, and reaction mixtures contained 0.2 ml of hemagglutinin, 0.2 ml of BS or antiserum, and 0.4 ml of chicken red blood cells (0.5%). Sera were treated by the trypsin-periodate method prior to HI tests (1).

Complement-fixation (CF) tests. CF titers were measured by the method of Fulton and Dumbell (2).

Rate zonal centrifugation. Performed 5 to 20%

sucrose gradients were overlaid with 0.5-ml samples. After centrifugation in an SW-39 head (Spinco; see text for details), ten 0.4-ml fractions were removed from a pin hole in the bottom of the tube. As standards for estimating sedimentation rates, solutions containing 10 mg/ml of crystalline bovine serum albumin were centrifuged in parallel with the samples.

Spectrophotometry. Ultraviolet (UV)-absorption spectra were obtained with a Spectronic 505 Recording Spectrophotometer (Bausch & Lomb). The spectra were corrected for light scattering by extrapolation from the nonabsorbing region.

RESULTS

UV absorption of solubilized proteins. The UV-absorption spectrum of the solubilized virus extract (after dialysis against 0.1 M acetic acid) gave a peak at 275 m μ and a minimum at 251 m μ . The ratio of absorbancy at 260 m μ to that at 280 m μ was 0.77, the spectrum conforming essentially to that expected for protein solutions.

Hemagglutinating and complement-fixing activity of envelope protein. Comparisons were made of the original virus concentrate, the insoluble residue from the chloroform-methanol extraction, and the residue solubilized with acetic acid. Lipid extraction diminished both activities of the virus concentrate. The HA titer was reduced to a trace while CF with PR8 antiserum was no longer detectable (Table 1). However, on solubilization with acetic acid, 20% of the HA titer was regained, and the CF titer equaled that of the original virus concentrate.

In a series of 10 experiments, examination of similar preparations confirmed these results. The hemagglutinating activity recovered varied from 2 to 20%, and CF titers ranged from 50 to 100% of the original virus concentrates.

Although the recovery of hemagglutinating activity demonstrated the presence of envelope protein in the preparations, it was conceivable that the inner nucleoprotein (s-antigen) contributed to the CF reaction with PR8 antiserum. To test this possibility, CF tests were performed with antibody specific for s-antigen. The solubilized protein did not react at a dilution of 1:10, while a control preparation of PR8 s-antigen was active to a titer of 640.

Antibody induction. To define further the properties of the solubilized protein(s), antigenic activity was assayed in animals. Two rabbits were inoculated with adjuvant emulsions containing 2,560 hemagglutinating units. Four weeks later, the sera of these rabbits had HI titers of 1,024 and 2,048 when tested with PR8 virus.

Infectivity. Preparations of solubilized proteins were tested in eggs for residual infectivity. Dilutions ranging from 10⁻¹ to 10⁻⁴, inoculated in 0.1-ml

TABLE 1. *Loss of activity after methanol-chloroform extraction and recovery on acetic acid treatment and dialysis*

Fraction	Hemagglutination titer	Complement-fixation titer
Virus concentrate.....	12,800	160
Lipid-extracted residue.....	40	<10
Acetic acid extract.....	2,560	160

volumes, did not lead to the formation of detectable hemagglutinin in a single passage.

Sedimentation characteristics of envelope proteins. On the basis of the general action of acetic acid on proteins, it would be expected that viral products of low molecular weight would be formed, and that subsequently the subunits would reaggregate when the acetic acid was replaced with a solvent of neutral pH. Therefore, a comparison was made of the sedimentation of envelope protein(s) in 0.1 M acetic acid and in BS. For this purpose, a lipid-extracted virus concentrate was dissolved in 67% acetic acid and dialyzed against 0.1 M acetic acid. A sample was dialyzed further against BS. A 2-ml amount of each sample was centrifuged at 35,000 rev/min for 2 hr in a Spinco model L ultracentrifuge (no. 40 head). The sediments were taken up in 0.1 M acetic acid and BS respectively, turbidity being noticeable only in the BS sample. The 0.1 M acetic acid sediment and supernatant fluid were now dialyzed against BS, and all four samples, after adjusting volumes to give concentrations equivalent to that of the original virus concentrate, were tested for HA and CF titers (Table 2).

The envelope protein(s) in 0.1 M acetic acid was nonsedimentable, all activity being found in the supernatant fluid. In contrast, when the material was suspended in BS, the greater part of the hemagglutinating activity was found in the pellet. Some CF antigen was sedimented, but the distribution of this antigen did not correlate with that of the hemagglutinin, suggesting the presence of a nonhemagglutinating CF antigen in the supernatant fluid.

More precise evidence was sought by subjecting similar preparations to rate zonal centrifugation. The samples were layered over sucrose gradients prepared with 0.1 M acetic acid and BS. After centrifugation for 3 hr at 35,000 rev/min, samples were collected, and those in 0.1 M acetic acid were dialyzed against BS prior to the HA test (Fig. 1). The sedimentation profile of the envelope protein(s) in 0.1 M acetic acid was characterized by a single, slow-moving peak, indicative of an active substance of uniform size and low sedimentation

TABLE 2. Ultracentrifugation at $100,000 \times g$ for 2 hr of envelope proteins of PR8 virus suspended in 0.1 M acetic acid and BS

Suspending medium during centrifugation	Fraction	Hemagglutination titer	Complement-fixation titer
Acetic acid, 0.1 M	Supernatant fluid	5,120	320
	Sediment	<10	<10
Buffered saline	Supernatant fluid	640	320
	Sediment	2,560	80
Virus concentrate		51,200	320

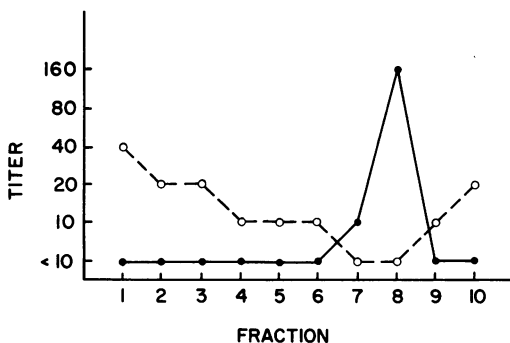


FIG. 1. Rate zonal centrifugations of viral envelope protein(s) through sucrose gradients for 3 hr at 35,000 rev/min. Extracts are suspended in 0.1 M acetic acid (●) and in buffered saline (○). Titer represents the HA titer on a logarithmic scale and fractions are the successive samples, no. 1 being lowest in the tube.

constant. The profile of the protein(s) in BS gave no indication of a prominent peak, but rather the activity was distributed throughout the fractions, with a major part being completely sedimented. Similar distributions were found if the conditions of centrifugation were reduced to runs of 30 and 60 min at 25,000 rev/min.

The sedimentation rate of the envelope protein(s) in 0.1 M acetic acid was determined by centrifugation for 20 hr at 35,000 rev/min and comparison of the profile with that of bovine serum albumin in the same run (Fig. 2). The sharpness of the single peak compares favorably with that of the serum albumin, indicating a high degree of homogeneity. The rate of sedimentation was slightly less than that of serum albumin, an approximate sedimentation constant of 4S being projected.

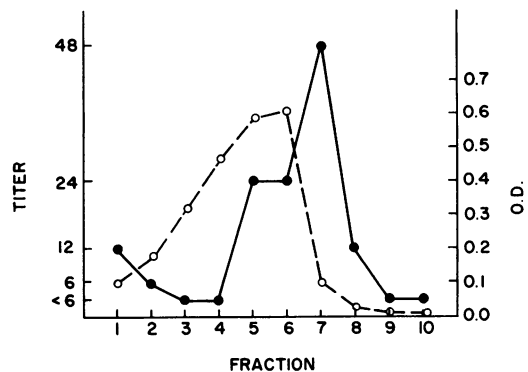


FIG. 2. Rate zonal centrifugation of viral envelope protein(s) in 0.1 M acetic acid (●) through a sucrose gradient for 20 hr at 35,000 rev/min. For comparison, a parallel run of bovine serum albumin (○) is included. Both the HA titer of the envelope protein(s) and the optical density of the bovine serum albumin are plotted on a linear scale.

DISCUSSION

The morphology of influenza virus is characterized by the presence of a filamentous inner component and a surrounding surface envelope. The investigations of Morgan et al. (8) of the ultrastructure of the influenza virus-infected cell disclosed the presence of viral envelope antigen at the cell surface and suggested the completion of the virus particle at this site and a similarity between the structures of the viral envelope and the infected cell membrane. Other studies of the nature of the viral envelope have been concerned with substructures obtained on disruption of the virus particle. Thus, Hoyle et al. (4), using ether treatment, split the viral coat into fragments with a rosettelike form consisting of surface spikes attached radially to pieces of envelope. Mizutani and Mizutani (7) reported on the physical properties of a hemagglutinating substructure derived from influenza virus by sodium deoxycholate treatment.

The present investigation is concerned with the structural and antigenic elements of the virus envelope at a lower level of organization, that of the polypeptides in various states of association. The study demonstrates that extraction with lipid solvents led to disruption of the complex lipid-protein organization of the virus envelope and, at the same time, the protein structure was modified, resulting in loss of complement-fixing reactivity. On solubilizing the residue with acetic acid, the envelope protein(s) dissociated into subunits with a sedimentation constant of 4S in 0.1 M acetic acid, suggesting a molecular weight of the order of 50,000. In neutral buffered saline, the subunits

reassociated into heterogeneous, larger masses, and greatly increased antigenic and hemagglutinating activity was detectable. In an earlier investigation, Laver (6) used detergent disruption to obtain low molecular weight subunits of influenza virus. Some of the antigenic and biological characteristics were retained, depending on the strain of virus. The relation of these subunits to those obtained with acetic acid cannot be fully evaluated at this time owing to the different analytical criteria measured. However, since in general the state of the subunits obtained on protein dissociation is dependent on the nature of the dissociating agent, it would be unlikely that the products of disruption with detergents would be identical to those obtained in the present report.

A major point concerns the influence of the physical arrangement of viral antigens on their antigenic specificity, in particular with reference to the effect of dissociation and association. In the present case, the recovery of antigenicity, as demonstrated both by complement fixation with antiserum to influenza virus and the induction of viral hemagglutination-inhibiting antibodies in experimental animals, indicated that the antigenic units of the reassociated protein(s) are similar to those of the original antigens present on the virus surface. In the same manner, antisera prepared with the detergent split products of the avian myeloblastosis virus reacted specifically with the antigens of the intact homologous virus (2). In contrast, tobacco mosaic virus was found to undergo alterations in its antigenicity during stages of its dissociation and reassociation. Rappaport (9) concluded, in this case, that the conformation of the polypeptides may be dependent on the nature of their aggregation and that this, in turn, influences antigenic specificity. A similar example is provided by poliovirus, in which antigenic specificity is dependent on the arrangement of the subunits in the capsid and also is altered by dissociation with guanidine (11).

Although there is definite serological cross-reaction between the hemagglutinin of intact virus and the solubilized envelope proteins, further experimentation is necessary to determine whether the antigens and the derived antisera are identical in their specificities. In view of the multiplicity of strains of influenza A virus and the proposal of Francis (3) that rearrangement of common antigenic elements is the basis of influenza virus variation, the dissociation and reassociation of influenza virus surface antigens could provide an

experimental basis for studying the minimal units responsible for virus antigenicity and their involvement in antigenic variation, possibly by rearrangement.

ACKNOWLEDGMENTS

This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, U.S. Army, Washington D.C., and by the National Foundation.

LITERATURE CITED

1. DAVENPORT, F. M., AND E. MINUSE. 1964. Influenza viruses, p. 455-469. *In* E. H. Lennette and N. J. Schmidt [ed.], *Diagnostic procedures for viral and rickettsial diseases*, 3rd ed. American Public Health Association, Inc., New York.
2. ECKERT, E. A., R. ROTT, AND W. SCHÄFER. 1964. Studies on the BAI strain A (avian myeloblastosis) virus. I. Production and examination of potent virus-specific complement-fixing antisera. *Virology* **24**:426-433.
3. FRANCIS, T., JR. 1960. On the doctrine of original antigenic sin. *Proc. Am. Phil. Soc.* **104**:572-578.
4. HOYLE, L., R. W. HORNE, AND A. P. WATERSON. 1961. The structure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether. *Virology* **13**:448-459.
5. KATES, M., A. C. ALLISON, D. A. J. TYRRELL, AND A. T. JAMES. 1961. Lipids of influenza virus and their relation to those of the host cell. *Biochim. Biophys. Acta* **52**:455-466.
6. LAVER, W. G. 1964. Structural studies on the protein subunits from three strains of influenza virus. *J. Mol. Biol.* **9**:109-124.
7. MIZUTANI, H., AND H. MIZUTANI. 1965. Small hemagglutinin of influenza virus and its responses to antibodies and inhibitor. *Virology* **26**:761-763.
8. MORGAN, C., R. A. RIFKIND, K. C. HSU, M. HOLDEN, B. C. SEEGAL, AND H. M. ROSE. 1961. The application of ferritin-conjugated antibody to electron microscopic studies of influenza virus in infected cells. I. The cellular surface. *J. Exptl. Med.* **114**:825-832.
9. RAPPAPORT, I., A. SIEGEL, AND R. HASELKORN. 1965. Influence of the state of subunit aggregation on the antigenic specificity of TMV and TYMV. *Virology* **25**:325-328.
10. REITHEL, F. J. 1963. The dissociation and association of protein structures. *Advan. Protein Chem.* **18**:123-226.
11. SCHARFF, M. D., J. V. MAIGEL, AND L. LEVINTOW. 1964. Physical and immunological properties of a soluble precursor of the poliovirus capsid. *Proc. Natl. Acad. Sci. U.S.* **51**:329-337.