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Envelope Protein of Influenza Virus

I. Hemagglutinating Activity of Reassociated Subunits

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The hemagglutinating properties of influenza virus envelope protein, prepared by reassociation of polypeptide subunits, have been defined and compared with those of virus and ether-split hemagglutinin. In general, the characteristics of the intact and ether-split virus were found to be similar, whereas those of the envelope protein were distinctly different. The use of chicken, pigeon, and guinea pig erythrocytes both at 23 and 4 C disclosed that the hemagglutinating titers of envelope protein preparations were particularly dependent on the system employed. Under optimal conditions, with guinea pig cells at 4 C, the titers of envelope protein preparations were equivalent to those of the original virus concentrates. The hemagglutinating activity of envelope protein was particularly sensitive to elevated temperature, concentrated urea, sulfhydryl-reducing reagents, and tryptic digestion at high salt concentrations. In all these respects, the intact virus was more resistant than the envelope protein. Interpretation of the data indicates that the hemagglutinin is stabilized when associated with the lipid micelle at the surface of the virus.

The influenza virus particle consists of an inner core of ribonucleoprotein surrounded by a lipid-rich envelope with projecting spikes. Two functionally and physically distinct proteins have thus far been demonstrated to be associated with the viral envelope. The enzyme, neuraminidase, has been separated from the envelope by the action of proteolytic enzymes (9, 10). A second protein, referred to as envelope protein, was isolated by extraction with protein dissociating reagents (5, 6). Its functions include responsibility for hemagglutinating activity, contributing to the strain-specific antigenicity of the virus, and acting as a structural component of the viral envelope, particularly if not solely in the organization of the surface spikes.

Derivatives of envelope protein have been isolated by extraction of denatured viral protein with either of two dissociating reagents: acetic acid or concentrated urea with dithiothreitol. Both methods reduce the protein to 2*S* polypeptide subunits, but these differ in their manner of reassociation. The serological and physical evidence, as of now, suggests that the same antigenic envelope protein is involved, but that the reassociated derivatives exist in two different physical forms. In both cases, the serological properties are similar to those of the surface antigens of the intact virus, but the smaller uniform product resulting from urea-dithiothreitol extraction neither adsorbed to nor agglutinated erythrocytes (6). In contrast, the subunits obtained by the acetic acid procedure reassociated to a greater extent, the resulting larger protein macromolecules being variable in size as determined by sedimentation and capable of agglutinating erythrocytes (5).

This investigation is a continuation of the study of the properties of the envelope protein of influenza virus strain PR8, with particular emphasis on the hemagglutinating macromolecules obtained by acetic acid extraction. By comparing the activity of envelope protein in its isolated reassociated state with that in its native state as part of the envelope of intact virus, aspects of the role of structural organization on hemagglutinating activity can be evaluated. In certain cases, comparison is also made with ether-split virus (7), in which state some of the viral lipid is presumed to be extracted and activity is associated with fragments resembling morphologically the viral envelope.

MATERIALS AND METHODS

Virus. Influenza virus strain PR8 concentrates were prepared from fluids of infected embryonated eggs by adsorption-elution first with chicken red cells, then with $BaSO_4$ (4), followed by two cycles of low and high speed centrifugation (6300 × *g* for 10 min and 26,340 × *g* for 1 hr). The virus was suspended in 0.15 M NaCl to give concentrates containing an average of 200,000 hemagglutinating units per ml.

Envelope protein. The preparation, described fully

in an earlier report (5), involved extraction of the virus concentrate with methanol-chloroform, solubilization of the denatured protein in 67% acetic acid, and stepwise dialysis into buffered saline (BS; 0.02 M phosphate buffer, *p*H 7.4, and 0.15 M NaCl).

Ether-split hemagglutinin. One volume of virus concentrate in BS was shaken vigorously at 4 C for 30 min with 2 volumes of ether. After centrifugation at 2,000 rev/min for 10 min, the water phase was collected and soluble ether was blown out with a stream of nitrogen.

Hemagglutination (HA) tests. Standard procedures with use of plastic trays were used. Reaction mixtures contained 0.2 ml of the serial dilutions of hemagglutinin, 0.2 ml of BS, and 0.4 ml of 0.5% red blood cells. Routine tests with chicken erythrocytes were performed at room temperature, whereas those with guinea pig cells were at 4 C.

Neuraminidase test. The procedure of Warren (11) was followed with *n*-acetyl neuraminylactose as substrate. Neuraminidase activities are expressed in terms of changes in optical density.

Rate zonal centrifugation. Preformed 5 to 20% sucrose gradients in 0.02 M tris(hydroxymethyl)-aminomethane (Tris) chloride (*p*H 8) and 0.15 M NaCl were overlaid with 0.5-ml samples. After centrifugation in an SW-39 head (Spinco), 10 fractions of 0.4 ml were removed from a pinhole in the bottom of the tube.

Enzyme treatments. Preparations of envelope protein were incubated with enzyme for 30 min at 37 C, then diluted 1:3 with chilled buffered saline and tested for HA. The final concentrations of the reaction mixtures were: (i) crystalline trypsin, 2 mg per ml of 0.05 M phosphate buffer (PB), *p*H 7.5, and 0.05 M Tris chloride, *p*H 8; (ii) pronase, 2 mg per ml of 0.05 M PB, *p*H 7.5; (iii) chymotrypsin, 2 mg per ml of 0.05 M PB, *p*H 6.3; (iv) papain, 10 mg per ml of BS and 0.01 M cysteine; and (v) ribonuclease, 2 mg per ml of BS.

RESULTS

Influence of conditions of HA test. As described in a previous paper (5), the HA titers of envelope protein preparations were measured by use of chicken cells at room temperature. Despite the recovery of essentially all complement-fixing activity when compared with the original virus concentrates, recovery in terms of HA titers was low. This discrepancy suggested that either a large part of the envelope protein was in a non-hemagglutinating form or that the hemagglutinating properties of isolated protein and intact virus were different. The latter possibility suggested that changes in the conditions of the HA test result in altered ratios of the activities of protein and virus.

To examine this question, the HA titers of envelope protein preparations were measured with chicken, guinea pig, and pigeon red blood cells at room temperature and at 4 C. Titers were compared with those of the original PR8 virus concentrate and its ether-split viral hemagglutinin derivative. Marked differences in the HA titers were disclosed for the three hemagglutinating substances (Table 1). The activity of the virus concentrate was least dependent on the type of erythrocyte or temperature. The ether-split viral hemagglutinin reacted to highest titer with guinea pig cells and lowest with pigeon cells. However, most striking was the wide variation in the characteristics of the envelope protein preparation. In this case, titers were increased greatly both by use of guinea pig cells and also by the lower temperature.

TABLE 1. *Comparison of the hemagglutinating titers of PR8 influenza virus, ether-split virus, and envelope protein with chicken, pigeon, and guinea pig erythrocytes measured at room temperature (23 C) and 4 C*

Erythrocyte	Temp (C)	HA titers[a]		
		Virus concentrate	Ether-split virus	Envelope protein
Chicken	23	2^{11}	2^{10}	2^{4}
Pigeon	23	2^{11}	2^{7}	$<2^{1}$
Guinea Pig	23	2^{12}	2^{12}	2^{6}
Chicken	4	2^{12}	2^{10}	2^{6}
Pigeon	4	2^{12}	2^{7}	2^{5}
Guinea Pig	4	2^{12}	2^{12}	2^{12}

[a] All samples were derived from the same virus pool.

It is significant that the titer of the isolated envelope protein approximated that of the original virus concentrate when both were measured with guinea pig cells at 4 C. This indicates that there is essentially complete recovery of HA in the protein extract, agreeing with the results found for complement-fixing antigen, but that the nature of the binding to red cells is different for protein and virus as disclosed by the influence of species of red blood cell and temperature.

The results with erythrocytes of different species suggest that the size of the hemagglutinating particle, that is, virus as compared to the much smaller particulate protein, plays a determining role in HA. Further evidence was sought in a study of the hemagglutinating activity of different fractions obtained by physical separation from envelope protein preparations. As demonstrated in an earlier report (5), the repolymerized hemagglutinating protein consisted of a population of particles of varying dimensions resulting in a broad sedimentation profile. To determine whether fractions of this population reacted equally well with chicken and guinea pig cells,

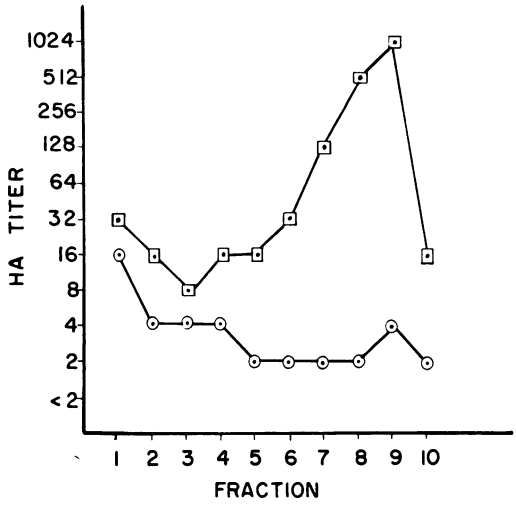

FIG. 1. *Rate zonal centrifugation of envelope protein in a sucrose gradient at 25,000 rev/min for 30 min, fraction 1 representing the bottom of the tube. Titers were measured with chicken (⊙) and guinea pig (⊡) erythrocytes. The samples measured with the latter cells were diluted an additional 40-fold in the HA test.*

an envelope protein preparation was centrifuged in a sucrose gradient at 25,000 rev/mn for 30 min. Ten fractions were collected and compared for HA titers with both kinds of erythrocytes, the samples being diluted an additional 40-fold for the measurements with guinea pig cells. The HA titers with guinea pig cells were consistently higher (Fig. 1), ranging from 80 to 10,240 times greater. Even more striking was the marked difference in the shapes of the sedimentation profiles as determined by the type of erythrocyte used. The hemagglutinin sedimenting more rapidly reacted better with chicken cells, whereas the slower sedimenting protein had disproportionately higher titers with guinea pig cells. The peak with guinea pig cells, in fraction 9, was not found in the profile measured with chicken cells. It should be noted that this particular envelope protein preparation had an unusually high peak of activity in the slower sedimenting fractions. The conclusion can be drawn that, not only did the envelope protein react to a higher titer when guinea pig cells were used, but that within the protein population the titer with guinea pig cells increased relative to that with chicken cells as the size of the polymer decreased.

Adsorption-elution and neuraminidase activity. A second aspect of the reaction of envelope protein with erythrocytes concerns its ability to absorb to and elute from the red cell surface. A comparison was made of the adsorption-elution of a virus concentrate and the derived envelope protein preparation with graded amounts of chicken erythrocytes. Amounts of 1-ml of the preparations, both diluted 1:100 to give HA titers in the same general range, were added to packed cells and the mixtures were held in an ice bath for 1 hr. The cells were centrifuged in the cold and washed once with cold BS. Elution was carried out into 1 ml of BS at 37 C. Both adsorbed and eluted samples were tested for HA titers with guinea pig cells at 4 C.

TABLE 2. *Adsorption and elution of PR8 virus and the derived envelope protein by use of graded amounts of chicken erythrocytes*

Packed chicken cells (ml)	HA titers			
	Adsorbed		Eluate	
	Virus concentrate	Envelope protein	Virus concentrate	Envelope protein
0.1	$<2^1$	$<2^1$	2^3	2^9
0.05	$<2^1$	2^1	2^5	2^8
0.025	$<2^1$	$<2^1$	2^6	2^8
0.012	2^2	2^2	2^8	2^9
0.006	2^3	2^2	2^8	2^9
0.003	2^6	2^4	2^9	2^9
None	2^{10}	2^9		

When a sufficient volume of red cells was used (Table 2), complete reduction of the HA titer of the envelope protein preparations was accomplished by adsorption, despite the fact that the protein had a low HA titer with chicken cells. Comparison of the adsorption of intact virus and protein disclosed that the binding capacity of cells for both was very similar, since in each case 0.025 ml or more of packed cells removed essentially all hemagglutinin and below this level the degree of adsorption appeared similar. Elution of the envelope protein appeared to be complete, but, in contrast, at higher cell concentrations elution of intact virus was significantly less. The results suggest that, at elevated temperatures, the binding of envelope protein to the cell surface is less firm than that of the virus, a relationship in accord with the marked increase in the HA titers of envelope protein when measured at 4 instead of at 23 C.

The demonstration of the elution of envelope protein from erythrocytes raised the question of the presence of the viral-associated neuraminidase which inactivates red cell receptors. On the basis of evidence that HA and neuraminidase activity are readily dissociated (9), it would be expected that viral neuraminidase might not be incorporated into the reassociated hemagglutinating envelope protein. The possibility did persist,

TABLE 3. *Neuraminidase activities of envelope protein and original virus concentrate*

Virus concentrate		Envelope protein	
Amt (ml)	OD[a]	Amt (ml)	OD[a]
0.002	0.33	0.01	0.04
0.003	0.42	0.05	0.04
0.004	0.56	0.10	0.04
0.005	0.63		
0.01	>1.0		

[a] Activities were measured in terms of changes in optical density (OD).

however, that neuraminidase was carried over during the isolation procedure as a separate entity.

Two envelope protein preparations were compared with the original viral stock for neuraminidase activity. Although all three samples had equal HA titers of 160,000 per ml when tested with guinea pig cells, the envelope protein preparations gave no detectable neuraminidase activity (Table 3). Calculated on the basis of the sensitivity of the test, residual neuraminidase activity of the envelope protein was considerably less than 2% of that of the virus concentrate. The possibility that neuraminidase protein is present, although in an inactive state cannot be excluded, but the evidence obtained with the analytical ultracentrifuge of single peaks both with 2*S* and 4*S* preparations indicated that only one major protein was present in envelope protein preparations (6).

Inactivation of HA. Although the patterns of interaction with the surface of red blood cells disclosed some differences between the isolated protein and the intact and ether-split virus, further information was sought in a study of their stability in the course of various treatments known to alter the conformation of proteins or to reduce proteins to smaller entities. These treatments include thermal inactivation, enzymatic splitting of linkages, breaking with concentrated urea of the noncovalent bonds contributing to the association of polypeptides, and reduction of disulfide bonds by dithiothreitol (DTT).

To test the effect of elevated temperature, 1:200 dilutions of a virus concentrate and its derived envelope protein and ether-split hemagglutinin were held in a 56 C water bath. At intervals, samples were removed and stored in an ice bath. HA titers were measured by use of guinea pig cells at 4 C. Whereas neither intact virus nor ether-split hemagglutinin showed any significant loss of activity over a period of 1 hr, there was a rapid drop in the HA titer of the envelope protein preparation from 2^6 to 2^1 in 10 min (Table 4).

TABLE 4. *Thermal inactivation studies of the HA of influenza virus strain PR8, ether-split hemagglutinin, envelope protein at 56 C*

Time (min)	HA titer[a]		
	Virus	Ether-split virus	Envelope protein
0	2^7	2^8	2^7
5	2^8	2^7	2^2
10	2^8	2^7	2^1
20	2^8	2^7	2^1
30	2^9	2^7	2^1
60	2^8	2^7	$<2^1$

[a] Titers were measured with guinea pig cells at 4 C. All three forms of hemagglutinin were derived from the same virus concentrate.

Enzymatic digestion. A preparation of envelope protein was treated with a variety of enzymes for 30 min at 37 C as described under Materials and Methods. Whereas the control preparation had an HA titer of 2^7, trypsin, pronase, chymotrypsin, and papain all reduced the value to less than 2^1. In contrast, ribonuclease had no significant effect. The results confirm the protein nature of the viral hemagglutinin.

It has been demonstrated (2) that, in the presence of high concentrations of PB or NaCl, the HA of intact influenza virus strain PR8 was insensitive to the action of trypsin, whereas at low ionic strengths it was susceptible. By comparison of the isolated envelope protein with intact virus and ether-split hemagglutinin, it might be possible to determine whether the influence of ionic strength on tryptic digestion is mediated through a direct effect on the protein or whether high salt concentration stabilizes a structural organization in intact virus so that trypsin-sensitive linkages are shielded. In the following experiment, the influence of the ionic medium on tryptic digestion was tested with preparations of PR8 virus concentrate, ether-split hemagglutinin, and envelope protein. The results (Table 5) were markedly different depending on the form of the hemagglutinin. Although the isolated envelope protein was sensitive to the enzyme under all conditions, the intact and ether-split virus preparations lost their activity only in media of low ionic strength. In a separate experiment, 0.01 M $MgCl_2$ was shown to have an effect similar to that of 0.15 M NaCl. Higher NaCl concentration or the addition of low concentrations of divalent cations all protected the HA of the virus from tryptic digestion, but did not hinder enzymatic inactivation of the

TABLE 5. *Influence of ionic environment on the action of trypsin at 37 C for 30 min on the HA titers of intact virus, ether-split hemagglutinin, and envelope protein*

Hemagglutinin	HA titer[a]				
	Control	0.01 M Tris chloride (pH 8)	0.01 M phosphate buffer (pH 7.2)	0.1 M phosphate buffer (pH 7.2)	0.01 M phosphate buffer (pH 7.2) and 0.15 M NaCl
Virus	2^7	$<2^1$	$<2^1$	2^6	2^7
Ether-split virus	2^8	$<2^1$	$<2^1$	2^7	2^8
Envelope protein	2^6	$<2^1$	$<2^1$	$<2^1$	$<2^1$

[a] Measured with guinea pig cells at 4 C.

TABLE 6. *Action of DTT and urea on the HA of intact influenza virus, ether-split virus, and envelope protein*

Hemagglutinin	HA titer*		
	Untreated	DTT (0.5 mg/ml)	Urea 7 M
Virus	2^8	2^7	2^7
Ether-split virus	2^6	2^6	2^5
Envelope protein	2^8	$<2^1$	2^1

[a] All forms of the hemagglutinin were derived from the same virus concentrate. Treatment was for 1 hr at room temperature, and HA titers were measured with guinea pig cells.

isolated protein. Thus, in the case of the isolated envelope protein, the sites susceptible to the action of trypsin are exposed, whereas, in the native form, they are not available.

Action of urea and sulfhydryl reducing agents on virus and envelope protein. Concentrated urea in the presence of a sulfhydryl reducing agent, DTT, was demonstrated (6) to be an effective reagent for the solubilizing of denatured viral protein, yielding a nonhemagglutinating strain-specific envelope antigen. In the following experiment, urea and DTT were tested separately for their action on the hemagglutinating activity of intact virus, ether-split hemagglutinin, and isolated envelope protein. Urea, 8 M, in 0.05 M Tris chloride buffer (pH 8) was added to the sample to give a final urea concentration of 7 M. After 1 hr at room temperature the samples were diluted 40-fold with BS, and the HA titers were measured. The treatment with DTT was similar, the concentration of DTT being 0.5 mg/ml.

Neither concentrated urea nor DTT had a marked effect on the HA titer with guinea pig cells of the intact or ether-split virus (Table 6). In contrast, the treatment with either urea or DTT eliminated virtually all HA activity of the envelope protein. Samples of the treated protein taken at shorter time intervals demonstrated that the action of DTT was more abrupt. At 10 min, the DTT-treated sample had lost all HA, whereas the titers of the urea-treated protein declined gradually and residual activity was retained after 1 hr. The results indicate that, although the envelope protein HA was sensitive to both the dissociating action of concentrated urea and the exposure to free sulfhydryl groups, the hemagglutinin of the intact and ether-split virus was in a stabilized or protected form.

Viral derivative resulting from urea treatment of influenza virus. Dorman (3) demonstrated that short exposure of an A_2 strain of influenza virus to concentrated urea resulted in a drop in the HA titer when measured with chicken cells and in no change when guinea pig cells were used. The following results confirm and amplify his findings, indicating that a stable, DTT-sensitive substructure is produced by the action of concentrated urea on intact virus.

Urea and DTT, both separately and in concert, were tested for their action on the hemagglutinating activity of influenza virus. To samples of strain PR8 virus concentrate were added urea to a final concentration of 7 M, 0.7 mg/ml of DTT, and the combination of both reagents. The reaction mixtures were held at 23 C, and at intervals samples were removed and immediately diluted 1:20 with BS; the HA titers were measured with both guinea pig and chicken cells (Table 7). Urea alone effected a gradual decrease in HA titer when measured with chicken cells. DTT, in contrast, had no influence at all on the HA titers. The action of DTT was, however, marked when used in combination with urea, the HA titers measured with both kinds of cells falling off very rapidly.

When graded concentrations of urea, in the presence of 1 mg/ml of DTT, were tested for action on viral HA titer, concentrations as low as 5 M were equally effective, but there was no drop in HA titer on exposure to 3 M urea for 3 hr. DTT was effective at concentrations as low as 0.01 mg/ml in the presence of 5 M urea.

The question arose as to whether, during the course of such treatments, changes in the physical state of the virus particle paralleled the changes in hemagglutinating properties. Electron micrographs of samples exposed to 8 M urea for 3 min disclosed that essentially all virus particles were damaged in this interval. Erosion of the particles

TABLE 7. *Changes in HA titers of the PR8 strain of influenza virus on exposure to 7 M urea, 0.7 mg/ml of DTT, and the combined reagents at room temperature*

Time (min)	HA titer							
	Control		Urea		DTT		Urea-DTT	
	Chicken	Guinea pig	Chicken	Guinea pig	Chicken	Guinea pig	Chicken	Guinea pig
5	2^7	2^6	2^5	2^8	2^8	2^6	$<2^1$	$<2^1$
15	2^7	2^6	2^5	2^8	2^7	2^6	$<2^1$	$<2^1$
30	2^7	2^6	2^4	2^8	2^7	2^6	$<2^1$	$<2^1$
60	2^7	2^6	2^3	2^7	2^8	2^6	$<2^1$	$<2^1$
120	2^7	2^6	2^1	2^7	2^7	2^6	$<2^1$	$<2^1$

occurred, so that, although grossly damaged particles appearing like viral ghosts were recognizable, a large amount of fragmented viral substance was found. Recognizable products, such as the rosettes of ether-split hemagglutinin, were not observed. Thus, after such a short exposure time, essentially complete disruption of the virus particle was accomplished, although a high HA titer with guinea pig cells was retained.

Sedimentation profiles of urea-treated virus confirmed the disruption of virus particles. Virus was treated with 8 M urea for 5 min, then diluted fivefold to stop the reaction. The urea-treated suspension and a control virus suspension were centrifuged in a sucrose gradient for 30 min at 25,000 rev/min and the fractions were tested for HA titers. Whereas the HA of intact virus reached a peak at the bottom of the tube, the HA with guinea pig cells of the urea-treated virus was found in high titer in all fractions (Fig. 2). The results indicate that short exposure of virus to concentrated urea disrupts the virus particles, producing smaller, polydisperse hemagglutinating derivatives.

The sedimentation profiles of the urea-treated virus were noticeably different when determined with guinea pig or chicken cells. Not only were the titers lower when measured with chicken cells, but the shapes of the curves differed. In the lower half of the tube, the titers ran parallel, but, in the upper half, the titers with chicken cells fell off sharply while the curve obtained with guinea pig cells was attaining its peak. The results indicate that the larger, more rapidly sedimenting viral derivatives were capable of agglutinating both types of cells, whereas the less rapidly sedimenting material agglutinated only guinea pig cells. The dependence of the ratio of HA titers with the two species of erythrocytes on the size of the viral products is in accord with the results of a similar experiment with envelope protein, described in the first section of the Results.

The data suggest that the urea-split virus

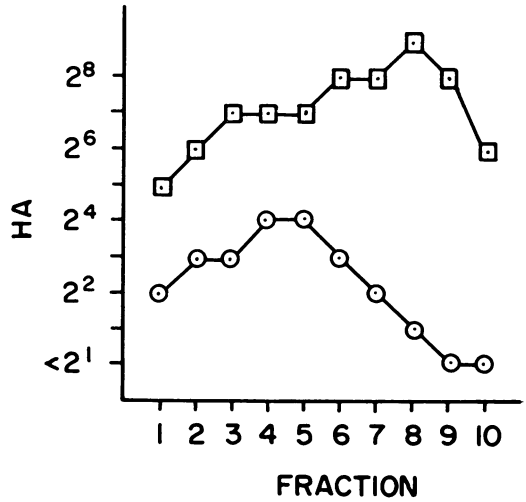

FIG. 2. *Rate zonal centrifugation of urea-split influenza virus in a sucrose gradient at 25,000 rev/min for 30 min, fraction 1 representing the bottom of the tube. Titers were measured with chicken (⊙) and guinea pig (⊡) erythrocytes.*

product is a stable derivative since prolonged exposure to concentrated urea did not result in significant loss of HA titer measured with guinea pig cells. However, when the urea was removed by dialysis, subsequent treatment with 1 mg/ml of DTT or with trypsin in the presence to 0.15 M NaCl for 30 min led to loss of all measurable HA. In both of these respects, the urea-split virus differed from the intact influenza virus particle.

DISCUSSION

The results of the present investigation demonstrate that quantitative recovery of viral hemagglutinins can be attained after extraction of viral lipid, denaturation of envelope protein, dissociation into protein subunits, and spontaneous reassociation to an active macromolecule. Although the envelope protein retains both its

virus-specific serological and hemagglutinating activities, differences in the structure of the isolated protein and the hemagglutinin present on the virus envelope lead to dissimilarities in the biological and physical properties of the two forms of hemagglutinin.

The envelope protein preparations represent the product of the repolymerization of polypeptide chains obtained by chemical dissociation of the denatured viral protein. This protein derivative is not homogeneous with respect to size, but rather the degree of polymerization appears to be indeterminate and a spectrum of protein molecules of varied dimensions results (5). Nevertheless, reassociated envelope protein does represent a particulate form of relatively low molecular weight viral hemagglutinin.

In contrast, the intact virus is a much larger structure over the surface of which are dispersed many sites responsible for hemagglutination. It is conceivable that these critical sites are localized on some 2,000 projecting spikes covering the viral envelope, both on the basis of the spatial availability of the spikes to the erythrocyte surface and the demonstration by electron microscopy that virus specific antibodies attached to the tips of the spikes (8). The ether-split hemagglutinin consists of fragments of envelope of variable sizes, the larger of which, the rosettes, are morphologically similar to the envelope of intact virus. The properties of the ether-split product and intact virus evaluated in this study confirm the similarity.

The first notable difference in the properties of the various hemagglutinins is disclosed by their reactions with erythrocytes of various species, particularly chicken and guinea pig. The thesis has been proposed by Choppin and Stockenius (1) that, on continued ether-splitting of influenza virus leading to the production of smaller particles, the HA titers obtained with chicken cells decreased more markedly than those with cells from some other species. Dorman (3) also showed that exposure of influenza virus to concentrated urea resulted in a decrease in HA titer with chicken cells but not with guinea pig cells. Further evidence is provided in this study since, not only does the envelope protein have a much higher titer with guinea pig cells at 4 C than with chicken cells at 23 C, but, when envelope protein is fractionated by rate zonal centrifugation, those fractions containing the less rapidly sedimenting components were characterized by particularly high titers with guinea pig cells. A similar relationship holds on centrifugation of the product obtained by treatment of intact virus with concentrated urea. It can now be concluded on the basis of results with several distinct types of viral degradation products that, as the size of the particle decreases, the titer assayed with chicken cells falls off. This does not necessarily imply that inactive or "monovalent" structures are produced, since the hemagglutinating activity of the split products can be demonstrated with other cell systems. In this respect, it should be noted that the uniform, low molecular weight 4*S* antigen, produced by extraction of envelope protein with urea-DTT, neither adsorbed to red blood cells (6) nor agglutinated chicken, pigeon, or guinea pig cells at 4 or 23 C (*unpublished data*).

Studies of the stability of the hemagglutinating activity of virus and envelope protein under varied conditions provide further evidence of the differences in properties of the several forms of hemagglutinin. Exposure to elevated temperatures, high concentrations of urea, sulfhydryl reducing agents, and trypsin in the presence of high salt concentrations demonstrated, in all cases, that the hemagglutinating activity of the envelope protein was more labile than that of the intact virus or ether-split hemagglutinin. Increased sensitivity to such a wide range of experimental treatments suggests that the reassociated subunits are more loosely bound to each other than in the native state on the viral envelope. The most striking example is the absolute difference in the sensitivities of protein and intact virus to the sulfhydryl reducing agent, DTT. Prolonged exposure of virus to this reagent led to no significant change, whereas inactivation of the envelope protein hemagglutinin was both rapid and complete. The results indicate that the integrity of the reassociated protein is dependent on the persistence of disulfide linkages between the subunits, whereas, in the case of intact virus, other linkages are sufficient to stabilize the hemagglutinin or promote a configuration protecting the disulfide bonds.

The properties of the viral derivative obtained by exposing virus to concentrated urea provide evidence that distinctly different structural elements are susceptible to the action of concentrated urea and sulfhydryl reducing agents. Concentrated urea disrupts the virus into small stable particles which retain their ability to agglutinate guinea pig cells. Although this hemagglutinin resists further degradation by urea, its activity is completely sensitive to treatment with DTT. In respect to its sensitivity to DTT and trypsin in high salt concentrations, the urea-split virus resembles the envelope protein. However, resistance to inactivation of its hemagglutinating activity by further treatment with urea differentiates it from the envelope protein.

The greater stability of the hemagglutinating activity of intact and ether-split virus as compared

to envelope protein suggests a difference in molecular organization. A parallel is found in the assembly of the subunits of tobacco mosaic virus into either a stack-disc or helical structure. A conceivable mechanism for the stabilization of the hemagglutinin of intact virus might be found in the association of the active protein with the lipids of the virus envelope. The demonstration that high salt concentrations or low concentrations of divalent ions protect viral hemagglutinin but not envelope protein from tryptic digestion suggests that linkages split by trypsin are shielded in intact virus and exposed in isolated envelope protein. Stabilization could result from penetration of lipophilic protein side chains into the viral lipid micelle, the structure of which is particularly susceptible to changes in ionic environment. Such an association would result in increasing the strength of bonding between subunits and possibly in steric protection of labile groups. However, any conformational changes are limited to the extent that neither major changes in antigenicity nor loss of hemagglutinating activity result.

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