Disruption of Myxoviruses with Tween 20 and Isolation of Biologically Active Hemagglutinin and Neuraminidase Subunits

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Myxoviruses were disrupted with Tween 20 at high pH, and the major surface antigens were separated in biologically active form. The neuraminidase had a sedimentation coefficient of 10.8S, and the hemagglutinin had a sedimentation coefficient of 8.1S. Electron microscopic examination of negatively stained preparations revealed structures identical in size and morphology to the neuraminidase and hemagglutinin subunits described by others. Inhibition of neuraminidase activity by antibody to the hemagglutinin which occurred with intact viruses (probably for "steric" reasons) did not occur after the viruses were disrupted with Tween 20. Serological assays for neuraminidase were possible in the presence of the mild surfactant, whereas serological assays for hemagglutinin were possible after removal of the reagent. Disruption of myxoviruses with Tween 20 therefore provides a method for the independent study of these antigens during antigenic drift.

Myxoviruses can be disrupted with lipid solvents such as ether (5, 10, 18, 22) or by ether and Tween 80 (9, 21). Two components are obtained by disruption of virions in this way, the nucleocapsid or S antigen and the surface projections containing hemagglutinin and neuraminidase. Treatment of myxoviruses with sodium dodecyl sulfate (SDS) or deoxycholate (DOC; 14-17) liberates the hemagglutinin and neuraminidase subunits but results in destruction of the biological activity of one or both subunits in some virus strains.

Treatment of myxoviruses with Tween 20 under alkaline conditions is an efficient method for liberating intact nucleoprotein antigens from myxoviruses (8). The present communication shows that biologically active hemagglutinin and neuraminidase can be separated from myxoviruses after similar treatment. Serological assays of these antigens in the presence of the reagent is possible, allowing the independent serological study of neuraminidase activity in the presence of hemagglutinin.

MATERIALS AND METHODS

Viruses. The following type A influenza viruses were used in this study: (i) A₀/NWS, (ii) PR8, (iii) A₂/RI/5⁺ (3), (iv) A₂/Aichi/2/68, (v) A₂/Tokyo/3/67, (vi) recombinant X-7 (11), and (vii) recombinant X-15 (12). The paramyxovirus Newcastle disease virus (NDV) was also used.

The viruses were grown in the allantoic cavity of 11-day-old chick embryos at 35 C and purified by adsorption-elution on chick erythrocytes followed by differential centrifugation and then sedimentation through a sucrose gradient (16). A concentrated preparation of NDV was kindly provided by D. W. Kingsbury.

Isolation of neuraminidase from X-7 virus. The neuraminidase of A₂/RI/5⁺ virus was isolated from the recombinant influenza virus X-7 by disruption of virus with SDS and separation of neuraminidase from the other virus proteins by electrophoresis on cellulose acetate (12, 16).

Antisera. Hyperimmune rabbit antisera to the viruses and to the isolated neuraminidase were prepared as described previously (12).

Treatment of viruses with Tween 20. Virus preparations were dialyzed against 0.02 M bicarbonate buffer (pH 10.0), and equal volumes of virus and 1% Tween 20 in 0.02 M bicarbonate buffer (pH 10) were mixed. The mixture was held for 4 hr at 37 C and then dialyzed against 0.1 M phosphate buffer to neutralize the mixture. Residual intact virus particles were removed by centrifugation at 60,000 × g for 30 min. The Tween 20 was removed from the disrupted virus preparation by chromatography on a Sephadex G200 column (12 × 600 mm) with 0.05 M phosphate-buffered saline (pH 7.2) as eluant.

Serological methods. Hemagglutinin titrations and hemagglutinin inhibition assays were done in plastic trays (WHO type) by using chicken erythrocytes; neuraminidase titrations and neuraminidase inhibition tests were done with fetuin as substrate as previously described (23). Neuraminidase activity was

expressed as optical density (OD) readings at 549 nm. Fractions from gradients containing sucrose were dialyzed against physiological saline to remove the sucrose which interfered with the neuraminidase assay.

Antibody blocking test for hemagglutinin. To detect the presence of the nonhemagglutinating antigen in the presence of Tween 20, the following antibody blocking test was used. Four inhibiting doses of antibody to the hemagglutinin of the virus (in 0.025 ml) were mixed with serial twofold dilutions of the Tween 20-treated virus (0.25 ml) and allowed to react together for 30 min at room temperature. Four hemagglutinating doses of whole virus (in 0.025 ml) and 0.025 ml of 5% chicken erythrocytes were then added, and the test was read after 35 min at 23 C. Hemagglutination indicated the presence of antigen, and the end point of the test was recorded as for standard hemagglutination titrations.

Estimation of sedimentation coefficients. For zonal rate centrifugation, 5-ml linear gradients (5 to 20% sucrose in 0.05 M phosphate buffer in 0.15 M NaCl, pH 7.2) and a swinging bucket rotor (Spinco SW 39) were used. Some of the gradients also contained 0.1% Tween 20. Samples were collected from the top of the tube with an ISCO density gradient fractionator and were assayed for neuraminidase and hemagglutinin after dialysis against phosphate-buffered saline to remove the sucrose. Sedimentation coefficients were estimated by the method of Martin and Ames (19) with human hemoglobin as the reference protein.

Electron microscopy. Influenza virus (X-7) was disrupted with Tween 20, and the surfactant was removed by chromatography as described above. Drops of this preparation were placed on carbon-coated grids and negatively stained by inverting onto 2.5% phosphotungstic acid (pH 7.2) for 1 min. The preparations were examined in a Siemens Elmiskop I electron microscope by use of double condenser illumination and 80 kv accelerating voltage. Isolated and concentrated neuraminidase from X-7 virus (12) was negatively stained in an identical manner and used as a reference.

RESULTS

Treatment of myxoviruses with Tween 20.

Treatment of influenza viruses at 23 C with Tween 20 and alkaline pH was found to be inefficient in disrupting influenza virus particles, confirming the observation of Hosaka (8). However, treatment of myxoviruses with Tween 20 at pH 10 at 37 C was effective in disrupting the virions (Table 1). Under these conditions, a marked or complete loss of hemagglutinin activity, with little or no loss in neuraminidase activity, resulted. The loss in hemagglutinin activity was accompanied by a loss in turbidity of the virus preparation. The hemagglutinin activity that remained after Tween 20 treatment could be removed by centrifugation or by absorption with chicken red blood cells with minimal loss of enzyme activity. These findings suggest that treatment of

TABLE 1. *Effect of Tween 20 on liberation of hemagglutinin and neuraminidase from certain myxoviruses*

Virus	Time of treatment	Hemagglutinin titer (log ₁₀)		Neuraminidase activity ^a	
		Unadsorbed	Adsorbed ^b	Unadsorbed	Adsorbed
Influenza X-7	hr				
	2	2.86	<1.60	0.358	0.275
	4	2.30	<1.60	0.320	0.268
	8	<1.60	<1.60	0.300	0.270
	Control (not treated)	4.27	<1.60	0.400	0.032
NDV	2	2.95	<1.60	0.635	0.468
	4	2.95	<1.60	0.590	0.527
	8	2.80	<1.60	0.460	0.450
	Control (not treated)	4.09	2.07	0.670	0.088

^a Neuraminidase values are the OD readings at 549 nm for 0.1-ml volumes of virus assayed as described.

^b Adsorption was done by mixing 0.2 ml of packed chicken cells with 1.0 ml of treated virus preparation at 0 C.

myxoviruses with Tween 20 results in a dissociation of the virus into hemagglutinin and enzyme subunits.

Separation of disrupted influenza virus from Tween 20 by column chromatography on Sephadex G200. Chromatography of the disrupted virus preparation on Sephadex G200 separated the detergent from the disrupted virions. Figure 1 shows that, after removal of the Tween 20, the hemagglutinin activity of the preparation could be detected by the conventional hemagglutination test and that under these conditions the hemagglutinin and the neuraminidase were eluted from the column as a single peak. The neuraminidase showed some heterogeneity and approximately 50% of the activity eluted after the hemagglutinin.

Adsorption of the samples from the Sephadex G200 column with chicken erythrocytes at 0 C quantitatively removed the hemagglutinin activity with minimal loss of the neuraminidase activity. This indicates that the neuraminidase is predominantly unassociated with the hemagglutinin after removal of Tween 20 and that the neuraminidase of this virus, as distinct from bacterial neuraminidase (1), is not absorbed by chicken erythrocytes.

Separation of the hemagglutinin and neuraminidase of Tween 20-disrupted influenza virus on sucrose gradients. The neuraminidase and hemagglutinin of Tween 20-disrupted influenza virus

FIG. 1. Separation of Tween 20 from disrupted influenza virus. A 0.5-ml amount of influenza virus X-7 (hemagglutinin titer, $4.30 \log_{10}$ units per ml) was treated with 0.5 ml of 1% Tween 20 in 0.02M bicarbonate buffer (pH 10) for 4 hr at 37 C and centrifuged at $100,000 \times g$ for 30 min to remove intact virus particles. The supernatant fluid (0.2 ml) was applied to a Sephadex G200 column (60×1.2 cm) equilibrated with phosphate-buffered saline (pH 7.2). The columns were eluted with phosphate-buffered saline (pH 7.2), and 1.0-ml samples were collected and assayed for hemagglutinin by the conventional method and for neuraminidase activity. The void volume of the column was determined by using *Escherichia coli* as marker and was 20 ml.

(X-7) were separated on sucrose gradients (Fig. 2). The hemagglutinin was very heterogeneous when centrifuged in the absence of Tween 20 (Fig. 2A) but showed a peak of biological activity. The neuraminidase was more homogeneous and sedimented more rapidly than the peak hemagglutinin activity. Human hemoglobin was centrifuged on an identical sucrose gradient in the same rotor to act as a sedimentation marker. The peak of hemagglutinin activity was estimated to have a sedimentation coefficient of 9.1S when compared with the hemoglobin standard, whereas the neuraminidase had a sedimentation coefficient of 11.7S.

The hemagglutinin activity of X-7 influenza virus could not be detected when disrupted virus was centrifuged on a sucrose gradient containing 0.1% Tween 20. The hemagglutinin subunits could, however, be detected by an antibody blocking test, and, under these conditions, the sedimentation coefficient of the hemagglutinin was 8.1S (Fig. 2B). Neuraminidase activity could be detected in the presence of Tween 20, and the sedimentation coefficient of the neuraminidase under these conditions was 10.8S. The lower sedimentation value for the hemagglutinin and for the neuraminidase in the presence of Tween 20 and the heterogeneity of the hemagglutinin

FIG. 2. Sedimentation of the hemagglutinin and neuraminidase of X-7 virus after treatment with Tween 20. (A) Linear sucrose gradient (5 to 20%) in phosphate-buffered saline (pH 7.2). (B) Linear sucrose gradient (5 to 20%) in phosphate-buffered saline (pH 7.2) containing 0.1% Tween 20. A 0.1-ml amount of X-7 virus, treated with 0.1 ml of 1% Tween 20 in 0.02 M bicarbonate buffer (pH 10) for 4 hr at 37 C, was applied to a 5-ml linear sucrose gradient (5 to 20%) in phosphate-buffered saline (pH 7.2) and centrifuged at 35,000 rev/min for 15 hr at 4 C in a SW 39 rotor. Samples were collected from the top of the gradient, diluted 10-fold in saline, and dialyzed against saline to remove sucrose. Neuraminidase and hemagglutinin activities were estimated as described. Hemagglutinin activity could not be estimated in the presence of Tween 20, and the hemagglutinin antigen was detected by an antibody blocking assay. Human hemoglobin was centrifuged in a companion tube as marker and was assayed by measuring its optical density at 412 nm.

in the absence of Tween 20 may be due to aggregation of the respective antigens in the absence of the surfactant.

Electron microscopic studies. Examination of the disrupted viral preparation revealed aggregates of particles (or polymers) in the shape of a rosette (Fig. 3A), approximately 30 to 35 nm in diameter. The individual knobs comprising the

FIG. 3. Negatively stained subunits of influenza virus. A, B, and C show Tween 20-disrupted X-7 virus, and D shows the isolated and concentrated neuraminidase. Arrows indicate polymers in each micrograph. (A) Rosette composed of about 10 neuraminidase subunits. $\times 210,000$. (B) Polymer of three hemagglutinin subunits. $\times 210,000$. (C) Copolymer of neuraminidase and hemagglutinin subunits. $\times 210,000$. (D) Isolated and concentrated neuraminidase; arrow indicates well-defined polymer. $\times 200,000$.

outer ring measured 8 to 9 nm at their greatest dimension. The size of the central structure was proportional to the number of subunits in the outer ring, and there was a suggestion of thin connecting strands between the central knob and the subunits of the outer ring. Another type of structure (Fig. 3B) was composed of varying numbers of rods measuring approximately 11 to 13 nm by 3 to 4 nm. The third structure (Fig. 3C) appeared to be composed of a combination of the subunits of the first two types. In addition to the structures described, there were large numbers of structures resembling the subunits of which the polymers were constructed. These were usually lying end-on or obliquely so that measurements of the individual subunits were not possible. The concentrated neuraminidase preparation (Fig. 3D) was identical in size and dimensions to the rosette structure illustrated in Fig. 3A.

Serological studies with Tween 20-disrupted myxoviruses: abolition of steric neutralization of neuraminidase activity by antibody to hemagglutinin. Neutralization of the neuraminidase activity on intact influenza viruses by antibody directed against the hemagglutinin sites is believed to occur by steric inhibition. Table 2 shows that antibody to hemagglutinin of NWS virus can neutralize the neuraminidase activity of a recombinant influenza virus, X-7, which possesses the hemagglutinin from A₀/NSW virus and the neuraminidase from A₂/RI/5⁺ virus. This neutralization of neuraminidase activity by antibody to the hemagglutinin of the virus was abolished when the X-7 virus was disrupted with Tween 20, but the neuraminidase in the disrupted virus preparation was neutralized by antibody to the enzyme.

TABLE 2. Inhibition of neuraminidase activity on intact X-7 influenza virus and on Tween 20-treated virus with antibody

Prepn	Serum dilution causing 50% inhibition of neuraminidase activity ($\log_{10}$ /ml)		
	Anti-NWS	Anti-X-7	Anti-RI/5 ⁺
Intact X-7 virus ^a	3.90	3.60	4.50
Tween 20-treated X-7 virus	<1.60	3.40	4.50
Purified neuraminidase from X-7 virus	<1.60	3.80	4.85

^a X-7 is a recombinant influenza virus possessing the neuraminidase from RI/5⁺ virus and the hemagglutinin from NWS virus.

Neutralization of the neuraminidase activity of intact or Tween 20-disrupted A₂/Aichi/2/68, A₂/Tokyo/3/67, and A₂/RI/5⁺ viruses with antisera to the intact viruses was also carried out. The results showed that the neutralization titers obtained with disrupted viruses were lower than when intact viruses were used, suggesting that antibody to the hemagglutinin was causing some steric inhibition of enzyme activity.

Treatment of crude virus preparations. As the majority of the work was done with purified X-7 virus, it was decided to determine whether biologically active subunits could be obtained from crude virus preparations. The viruses were concentrated approximately 10-fold from allantoic fluid by one cycle of high-speed centrifugation and then treated with Tween 20.

Table 3 shows that treatment of crude virus concentrates of A₀/NWS, A₂/RI/5⁺, and X-15 with Tween 20 did not disrupt all of the particles

TABLE 3. *Treatment of crude influenza virus preparations with Tween 20*

Virus	Assay	Un-treated virus	Virus treated with Tween 20	Virus treated ^a with Tween 20 and centrifuged
A ₀ /NWS	Hemagglutinin ^b	4.39	3.85	<1.60
	Neuraminidase ^c	0.148	0.058	0.058
A ₂ /RI/5 ⁺	Hemagglutinin	4.75	3.70	<1.60
	Neuraminidase	0.841	0.649	0.476
X-15 ^d	Hemagglutinin	4.60	4.09	4.12
	Neuraminidase	0.499	0.341	0.276

^a Tween 20-treated virus was centrifuged at $60,000 \times g$ for 30 min, and the supernatant fluid was tested for biological activity.

^b Hemagglutinin activity was measured in the conventional test.

^c Neuraminidase values are the OD readings at 549 nm for 0.1-ml volumes of virus assayed as described.

^d Recombinant containing equine/1/hemagglutinin RI/5⁺ enzyme.

but biologically active subunits were obtained. It was necessary to centrifuge the treated preparations at high speed ($60,000 \times g$ for 30 min) to remove nondisrupted virus particles. The hemagglutinin activity of A₀/NWS and A₂/RI/5⁺ could not be detected in the presence of the surfactant, whereas the hemagglutinin of X-15 virus could be detected in its presence. Crude virus preparations of A₀/PR8, A₂/Tokyo/67, and A₂/Aichi/68 were also disrupted with Tween 20; after high-speed centrifugation, hemagglutinin activity could not be detected in the presence of the surfactant but enzyme activity could be detected. X-15 virus differed from the other viruses tested in this study (NWS, PR8, RI/5⁺, A₂/Tokyo/67, A₂/Aichi/68, X-7, NDV), since these viruses required the removal of Tween 20 before hemagglutinin activity could be detected.

DISCUSSION

When several strains of influenza A and NDV myxoviruses were disrupted with the surfactant Tween 20 at alkaline pH, the hemagglutinin and neuraminidase of the viruses could be released and obtained in biologically active form. After treatment with Tween 20 the hemagglutinin titer of most of the virus preparations was markedly decreased or abolished, but after the surfactant was removed from the preparation the hemagglutinin activity could be detected. The mechanism whereby the surfactant inhibits hemagglutination is not known, but the most likely

explanation is that the monomeric subunits of the hemagglutinin cannot aggregate red blood cells (17) although they can absorb to them.

The sedimentation coefficient of the neuraminidase subunit of myxoviruses lies in the range from 9 to 10S (7, 15, 17), and the present estimate of 10.8S is close to these values. The sedimentation coefficient of the hemagglutinin of influenza virus has been estimated to be 7.5S (17), and the present value of 8.1S is close to this value. The advantage of the present method for disrupting myxoviruses is that the sedimentation coefficients of both the major capsid antigens can be estimated on the same virus preparation.

Laver and Valentine (17) reported that copolymers of hemagglutinin and neuraminidase are formed when these isolated, purified antigens from two different strains of influenza A virus are mixed and the detergent is removed. Recent studies with purified antibodies to either the hemagglutinin or neuraminidase (R. G. Webster and W. G. Laver, *unpublished data*) show that the aggregates formed are indeed copolymers with antigen from each of the parent viruses. The structures seen on electron microscopic examination of the disrupted virus preparations are identical to those described by Laver and Valentine (17). The rosette forms correspond to the neuraminidase polymers. The rod-shaped polymers resemble the hemagglutinin polymers, and the copolymers are identical in size and morphology to the copolymers of neuraminidase and hemagglutinin described by the above authors. Although the resolution of the isolated subunits leaves a great deal to be desired, there is little question that Tween 20 disrupts the influenza virion and liberates subunits. It was difficult to find copolymers of hemagglutinin and neuraminidase in the electron micrographs. This observation supports the biological finding that adsorption of the hemagglutinin with chicken erythrocytes does not cause an appreciable reduction in neuraminidase activity.

The measurements of the size of the individual knobs on the neuraminidase subunit (8 to 9 nm) correspond exactly to the values published by Drzeniek et al. (6) for the diameter of the "ring-like structures" seen in their preparations of influenza neuraminidase. However, the dimensions obtained by these workers (6) for the nonaggregated neuraminidase and for the hemagglutinin differ from the values obtained by Laver and Valentine (17) and by ourselves.

The present study shows that the steric inhibition of neuraminidase activity by antibody to the hemagglutinin can be overcome by disrupting the virus particle with Tween 20. Antigenic drift

(2) occurs in influenza viruses, both in the hemagglutinin and in the neuraminidase antigen (20), and may be the explanation for the origin of viruses responsible for interpandemic outbreaks of clinical influenza. The relative importance of the neuraminidase antigen in this context has not been studied in any detail. The studies to date (20) have been done with intact virus particles, and the problem of steric neutralization of enzyme activity by antibody to the hemagglutinin (or vice versa) leaves these studies open to some questions. The present method of disrupting influenza virus particles with Tween 20 and obtaining biologically active hemagglutinin and neuraminidase offers a method for studying these antigens independently. The importance of studying both of the virus-coded antigens on the surface of influenza virus was clearly demonstrated during the outbreak caused by Hong Kong/68; the hemagglutinin of Hong Kong/68 virus was serologically distinct from the virus of the preceding year (A₂/Tokyo/3/67), whereas the neuraminidase antigens on both the Hong Kong/68 and Tokyo/67 viruses were similar if not identical (4; R. G. Webster, *unpublished data*).

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