Purification of Influenza Virus with Arcton 113

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Arcton 113 has proved effective in the removal of host membrane fragments which cosediment with influenza virus during differential centrifugation. The virus appeared morphologically undamaged by the procedure and retained its infectivity, neuraminidase, and hemagglutinating activity. Pyrogenicity, however, was considerably reduced.

Fluorocarbons, such as Arcton 113 or Freon 112 in *n*-heptane, have been used for the purification of several viruses, e.g., foot and mouth, vaccinia, poliomyelitis, rabies, mumps, influenza, echo, and Coxsackie (3, 4, 6, 7, 10). Specific removal of contaminating host material occurred regardless of the species of origin.

The mode of action of fluorocarbon is unexplained. With viruses like vaccinia, poliomyelitis, foot and mouth, echo, and Coxsackie, which do not contain lipid, it is probable that fluorocarbons selectively remove lipid-containing host membranes. However, the purification technique works successfully with the viruses of influenza and mumps, which contain lipid, although these viruses are more susceptible to inactivation by fluorocarbons than are the lipid-free viruses. The results reported by Hamparian et al. (6) for influenza virus promoted our experiments to assess the effects of Arcton 113 on this virus. The extent of purification was measured chemically in relation to biological activity, and structural modifications were assessed by electron microscopy and pyrogenicity. The latter was measured because the pyrogenicity of influenza virus is thought to be associated with the structural integrity of the viral lipids (13).

MATERIALS AND METHODS

Virus. X-31, a recombinant virus (8), was used throughout the work. It contained the hemagglutinin and neuraminidase of A₂/Aichi/2/68 but had the growth characteristics in eggs of A0/PR8/34.

Cultivation of virus. X-31 influenza virus ($10^{4.5}$ 50% egg infective doses) in 0.2 ml of peptone-saline containing polymixin B sulfate (175 IU/ml) and neomycin sulfate (70 IU/ml) was inoculated into the allantoic cavity of 11-day-old embryonated hens eggs. After incubation for 48 h at 35 C, the eggs were chilled and the allantoic fluid was harvested.

Virus intended for use in vaccine was inactivated with β -propiolactone and concentrated by differential centrifugation in a Sharples centrifuge, but to assess the effect of Arcton 113 on infectivity it was

necessary to concentrate live virus on a small scale.

Concentration of live virus. The virus was sedimented from allantoic fluid onto 300 ml of 60% (wt/wt) sucrose in phosphate-buffered saline (PBS) in a BXV zonal rotor. The rotor was centrifuged at $25,000 \times g$ for 3 h at +4 C. The fractions containing virus were pooled, diluted in PBS, and resedimented to remove sucrose. The pellet was resuspended in PBS containing 0.2% sodium azide, and the suspension was stored at +4 C.

Concentration of virus by differential centrifugation. Allantoic fluid (20 liters) containing inactivated virus was clarified at $15,000 \times g$ in a Sharples centrifuge at 20 liters/h to remove large particulate material such as cell debris. The clarified product was recentrifuged at $90,000 \times g$ at a flow rate of 10 liters/h. The pellet was resuspended in 250 ml of PBS.

Treatment with Arcton 113. Two volumes of Arcton was added to each volume of virus concentrate. The mixture was agitated on a reciprocal shaker at 200 oscillations/min for 30 min at +4 C. The aqueous and organic phases were separated by centrifugation at 2,500 rpm for 10 min into an upper aqueous layer containing virus and a lower Arcton layer. For multiple extraction techniques, the aqueous layer was removed and re-extracted with 2 volumes of fresh Arcton.

Electron microscopy. Samples were concentrated by sedimentation and resuspended in PBS. They were added to carbon-coated Formvar grids, and salts were removed by addition and removal of distilled water to the grid. Phosphotungstic acid (2.5%, wt/vol), pH 6.8, was used as a negative stain.

Hemagglutination assay. The hemagglutination assay was titrated according to the World Health Organization recommendations (15).

Infectivity. Serial 10-fold dilutions were made in peptone-saline containing penicillin (500 U/ml) and streptomycin (500 μ g/ml). Four 11-day-old embryonated hen eggs were used for each dilution.

Each egg was inoculated with 0.2 ml of sample by the allantoic route. The eggs were incubated for 48 h at 35 C, and the allantoic fluids were harvested. The infectivity was calculated by the method of Reed and Muench (11) from the allantoic fluids (0.25 ml), which showed positive agglutination when shaken gently with an equal volume of chick erythrocytes (0.5, vol/vol, in PBS).

Neuraminidase assay. The method of Warren (14) was used to assay *N*-acetyl neuraminic acid released by the action of neuraminidase on fetuin. The incubation mixture consisted of 0.05 ml of fetuin (24 mg/ml of water), 0.05 ml of phosphate buffer (0.4 M, pH 5.9), 0.05 ml of saline (0.85%, wt/vol), and 0.05 ml of neuraminidase solution. The latter was diluted so that, after 4 h of incubation at 37 C, the *N*-acetyl neuraminic acid released gave a color reaction with an absorbance between 0.2 and 0.8 at 550 nm. The results were either reported as absorbance or were converted by comparison with a standard *N*-acetyl neuraminic acid graph to micromoles of *N*-acetyl neuraminic acid released per hour per milliliter of solution.

Phospholipid assay. Lipid was extracted from virus according to the method of Bligh and Dyer (2), using a final ratio of sample-methanol-chloroform of 0.8:2:1. The lipid was extracted finally into chloroform. This was evaporated to dryness in a water bath, and the lipid phosphorus was oxidized to organic phosphate by perchloric acid. Inorganic phosphorus was then assayed by formation of a blue phosphomolybdate complex based on the method of Allen (1). The method was modified slightly to increase the sensitivity. After evaporation of chloroform, perchloric acid (1 ml) was added to the combustion tube, which was carefully heated until the solution was clear. In the samples that contained a large amount of carbon, the digestion time was approximately 30 min, and additional perchloric acid was required. For the samples containing less carbon, the perchloric digest became clear after about 7 min, and only 0.6 ml of perchloric acid was added initially. The final volume of perchloric acid after digestion was 0.4 ml.

Distilled water (1 ml) was added to each sample followed by amidol reagent (1% [wt/vol] 2,4-diaminophenol hydrochloride in 20% [wt/vol] sodium metabisulfite) (0.4 ml) and ammonium molybdate solution (8.3%, wt/vol) (0.2 ml). A blue color developed and was read at 610 nm within 5 to 30 min of molybdate being added. A set of standard phosphate solutions was always assayed in parallel with unknown samples. A linear response was obtained for solutions of phosphate containing between 0 to 14 μ g of phosphorus per ml. The upper absorbance reading at 610 nm was 0.7.

Protein assay. Protein was assayed by the

method of Lowry et al. (9), modified for the autoanalyzer. Bovine plasma albumin was used as a standard. Interference by Triton was eliminated by increasing the reaction temperature to 95 C.

Pyrogenicity. The test for pyrogens was carried out according to the *European Pharmacopoeia* (5).

RESULTS

Purification with Arcton 113. A virus concentrate was shaken four times with Arcton 113 as described in Materials and Methods. A sample was removed at each stage (A1 to A4) and assayed for infectivity, neuraminidase, hemagglutinin, protein, and lipid-phosphorus. The starting material and A4 were examined in the electron microscope for morphological change.

Table 1 shows that infectivity, neuraminidase, and hemagglutinating ability of the virus remained constant throughout the treatment. The protein and lipid-phosphorus levels, however, were considerably reduced and the hemagglutinating units (HAU/milligram of protein ratio increased from 1.9×10^5 to 4.7×10^5 . It was concluded from these results that Arcton had removed extraneous host membrane fragments from the virus preparation. It is possible that some of the lipid came from the virus particles. However, no morphological differences were observable in the virus particles after treatment with Arcton (Fig. 1).

Effect of Arcton 113 on pyrogenicity of influenza. Intact influenza virus is pyrogenic and the presence of extraneous lipoprotein has been shown to enhance the effect (12). Arcton treatment provided a simple means of removing host protein, and it was therefore of interest to measure the pyrogen response of a virus preparation before and after Arcton treatment.

Virus was sedimented from allantoic fluid in a Sharples centrifuge. The pellet was resuspended in pyrogen-free PBS and a sample was diluted to 5,000 HAU/ml (200 IU/ml). This was compared in a standard pyrogen assay with virus that had been shaken twice with Arcton

TABLE 1. Effect of Arcton 113 on the biological and chemical properties of X-13 influenza virus

Virus ^a	Assay					
	HAU/0.25 ml	Neuraminidase (μ mol/h/ml)	Infectivity ^b (EID ₅₀ /0.2 ml)	Protein (μ g/ml)	Lipid-phosphorus (μ g/ml)	HAU/mg of protein
Starting material	51,200	16	$10^{8.75}$	1,060	7.8	1.9×10^5
A1	51,200	15	$10^{7.50}$	634	4.6	3.2×10^5
A2	51,200	14	$10^{7.50}$	530	3.4	3.8×10^5
A3	51,200	15	$10^{8.00}$	454	2.0	4.5×10^5
A4	51,200	14	$10^{8.50}$	440	1.7	4.7×10^5

^a A1-A4, Number of times the virus was treated with Arcton 113.

^b EID₅₀, 50% egg infective dose.

FIG. 1. X-31 influenza virus particles (A) before and (B) after treatment with Arcton 113, showing morphological similarity.

113 and then diluted to 5,000 and 40,000 HAU/ml.

Arcton treatment increased the HAU/milligram of protein ratio from 3.25×10^4 to 1.05×10^5 . Figure 2 shows the mean increase of temperature in rabbits for these virus prepara-

tions. The virus treated with Arcton did not produce a temperature rise and was nonpyrogenic at 5,000 HAU (200 IU), but at 40,000 HAU (1,600 IU) it induced a rise in temperature similar to that produced by 5,000 HAU of untreated virus.

FIG. 2. Effect of purification of virus with Arcton 113 on pyrogenicity of a Sharples concentrate. The mean change in rabbit temperature is shown for an Arcton-treated virus concentrate at 5,000 HAU/ml (200 IU/ml) (X) and 40,000 HAU/ml (●) and for the untreated concentrate at 5,000 HAU/ml (○).

DISCUSSION

Virus concentrated from allantoic fluid by differential centrifugation contained extraneous membrane fragments from the host cells. Treatment with Arcton removed this material without affecting the hemagglutinin, neuraminidase, or infectivity of the virus. When examined in the electron microscope, the treated virus appeared to be morphologically intact and the preparation lacked the membrane fragments visible in untreated material. Thus Arcton treatment provided a simple way of purifying an influenza virus suspension.

The treated preparation was much less pyrogenic than the untreated virus. Whether this was due solely to removal of extraneous host protein is open to question. Another possibility is that the structural integrity of the viral lip-

ids, which are thought to be responsible for the pyrogenicity of whole virus (13), were altered by Arcton. The alteration, however, could not be shown in the electron microscope.

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