Preparation and Characterization of Influenza Virus Cores

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SUMMARY

Lipid-free influenza virus cores have been obtained by a three step procedure consisting of (a) treatment with proteolytic enzyme, (b) fixation with formaldehyde and (c) delipidization with saponin or deoxycholate.

Several reagents proved efficient in removing the virus lipids as judged by morphological features and increased buoyant density, but only cores prepared by means of sodium deoxycholate have been characterized closely. Ultrathin sections revealed round bodies (about 65 nm in diam.) delineated by a single dense track and with an internal structure very similar to that of the complete virus particles. They contained both the nucleoprotein and the M-protein and no lipids.

It is proposed to call the limiting structure which appears (3 to 4 nm thick in ultrathin sections) the core shell.

INTRODUCTION

During the last few years convincing evidence has accumulated that the M-protein of influenza virus (Kilbourne et al. 1972) forms a spherical shell 3 to 4 nm thick with a diam. of about 70 nm located between the virus membrane and the nucleocapsid (for reference see Schulze, 1973) rather than a matrix filling the interior of the virus particle as suggested by Laver (1973). A concept of the virus core emerged in a few papers (Schulze 1970; Skehel, 1971; Nermut, 1972) but up to now the structures have only been partially characterized. This might be the reason why others prefer to speak of a complex virus envelope comprising the spikes, the lipid bilayer and the so called M- or ‘membrane’ protein (Compans et al. 1972; Lenard, Wong & Compans, 1974). Purification of the cores thus appeared to be an important step in the studies of influenza virus architecture. The present paper describes attempts to remove the lipids from the influenza virus particle while preserving its native internal organization.

METHODS

Virus. Influenza virus A, strain PR-8 was grown in eggs and concentrated from the allantoic fluid by adsorption onto, and elution from, chicken red cells and further purified by centrifugation through a sucrose density gradient (10 to 40 %, w/v). The sucrose was removed by repeated washing with phosphate buffered saline (PBS; Dulbecco & Vogt, 1954). The pellets obtained after the last centrifugation were resuspended in distilled water

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and kept at -70 °C in 0.5 to 1 ml samples containing 3 to 6 mg of virus protein as estimated by the method of Lowry et al. (1951).

**Preparation of spikeless particles.** The spikes were removed by means of caseinase C (Reginster, 1965, 1966) a proteolytic enzyme produced by a streptomyces, purified and supplied by L. Dierickx (C.N.P.E.M. at the University of Liège). Eight vol. of virus suspension were mixed with one vol. of caseinase C (3 mg protein/ml) in distilled water and one vol. of either potassium phosphate buffer, pH 7.0 ionic strength 0.1, or one vol. of PBS and incubated at 36 °C for up to 24 h (see results).

**Fixation of spikeless particles** was carried out with 5 % (v/v) glutaraldehyde (Taab Laboratories, Reading, England), or 6 % (v/v) formaldehyde (May & Baker, London, England), or 0.5 % osmium tetroxide (Johnson-Matthey Chemicals Ltd, London, England) at 4 °C for 30 min (glutaraldehyde and formaldehyde), or for 60 min (osmium tetroxide). The fixatives were then removed by dialysis against PBS for 2 h.

**Delipidization: reagents and procedures.** Stock solutions of various reagents were prepared as follows: (a) phospholipase-A (snake venom from Ancistrodon piscivorus piscivorus, Sigma, London, England) 4 to 8 mg/ml in distilled water; (b) lysolecithin (Sigma, London, England), 2 to 10 mg/ml in 0.1 sodium acetate buffer at pH 5.6; (c) saponin (white, BDH, Poole, England), 50 mg/ml in distilled water; (d) lithium bromide, caesium chloride and lithium chloride (all from Hopkin & Williams, Chadwell Heath, England) 50 mg/ml in distilled water; (e) sodium deoxycholate (DOC; BDH, Poole, England), 10 % (v/v); (f) Diethylether, n-butanol, or chloroform-methanol (2:1, v/v) were applied at 4 °C and supplied by BDH (Poole, England). Phospholipase-A and saponin solutions were filtered through a millipore membrane (0.45 μm pore size) before use. Final concentrations, temperature and length of contact of the above solutions with the virus particles are specified in the Results and in Table 2.

**Density gradient centrifugation.** Treated virus material was layered on top of a continuous 15 to 60 % (w/v) K-Na tartrate gradient and centrifuged in a 3 × 5 ml swing-out rotor (MSE-50, SW 39) at 100000 g for 2 h. The equilibrium was reached usually after 90 min. Material from the bands (usually clearly visible) was collected with a syringe fitted with a blunt needle and the refractive index was measured. The relationship between the density of the K-Na tartrate solutions and their refractive index was established by weighing tartrate solutions (in PBS) of various concentrations.

**Chemical and serological methods.** Thin layer chromatography of lipids was carried out as described by Payne (1964). For polyacrylamide gel electrophoresis of polypeptides the samples were boiled for 5 to 10 min with SDS and mercaptoethanol according to Skehel & Schild (1971). The single radial diffusion technique was carried out as described by Schild, Henry-Aymard & Pereira (1972) and the complement fixation was performed according to Sohier et al. (1956).

**Electron microscopy.** Negative staining was carried out with 4 % (w/v) silicotungstate, pH 6.5, or 1 % (w/v) uranyl acetate, pH 4.4. For ultrathin sections the virus material was fixed with formaldehyde (see above) followed by 1 % (v/v) osmium tetroxide at 4 °C for 1 h, two washings with PBS, one washing with distilled water, dehydrated with 0.5 % (w/v) uranyl acetate in 25 % (v/v) ethanol for 30 min and the usual alcohol series before embedding in Spurr’s resin (Spurr, 1969). Sections were cut with an LKB ultramicrotome and post-stained with 0.5 % uranyl acetate for 30 min and lead citrate (Reynolds, 1963) for 40 s. Specimens were observed with a Philips EM-300 electron microscope operating at 60 or 80 kV.
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RESULTS

Effects of caseinase C on virus particles

Most virus particles lost their spikes after the first 4 h at 36 °C, but about 10 to 20 % still possessed them and therefore the treatment was prolonged up to 24 h. Samples were then controlled by negative staining which revealed that about 40 to 50 % were smooth spikeless particles. The remaining particles were either penetrated by the stain or disrupted to various degrees. They often displayed a round ‘central body’ separated from fragments of the virus membrane by a narrow space filled with the negative stain (Fig. 1). The ‘central bodies’ were usually granular in appearance. The size of the granules ranged from 3 to 5 nm. Up to 20 % of the particles penetrated by the stain had their external membrane widely opened and their core was disrupted, sometimes releasing strands reminiscent of the nucleoprotein. Preparations negatively stained with uranyl acetate contained a lower percentage of disrupted particles, suggesting that at least some particles were disrupted on the grids during negative staining with silicotungstate. Ultrathin sections of spikeless particles provided a typical image of influenza virus but without the fringe of spikes (Kendal, Apostolov & Belyavin, 1969; Nermut, 1972). Staining with uranyl acetate before dehydration (Compans & Dimmock, 1969; Nermut, 1972) enhanced electron density of the outer track so that an asymmetrical virus membrane was clearly seen.

Effects of delipidizing reagents on unfixed particles

The spikeless particles were always used as a starting material in our attempts to remove the virus membrane without destroying the internal organization of the virion. A wide range of reagents was tried either singly or in various combinations. Their effect was either too mild (lysolecithin, phospholipase A, salts of casein or lithium) so that the virus membrane was not removed completely (monitored by electron microscopy and buoyant density) or too drastic (ether, Nonidet P-40, DOC, n-butanol or chloroform-methanol) so that particles were entirely disintegrated. It appears necessary, therefore, to fix the virus particles before applying the delipidizing reagents.

Effects of delipidizing reagents on fixed particles

We first tried glutaraldehyde because of successful experiments reported by Schulze (1970, 1972) who treated glutaraldehyde fixed WSN virus with Nonidet P-40. However, as with influenza virus X-31 (Nermut, 1972) we were not successful with the strain PR-8 either. Therefore we tested other fixatives such as formaldehyde and osmium tetroxide. Formaldehyde preserved the internal virus structure better than osmium tetroxide and was therefore used in further attempts to isolate and purify the ‘central bodies’. The main delipidizing procedures applied to formaldehyde fixed spikeless particles as listed in Table I. Their efficiency was monitored by electron microscopy after negative staining and ultrathin sectioning, by thin-layer chromatography of lipids and by estimation of the buoyant density of the recovered particles. It should be mentioned that treatment with organic solvents such as n-butanol or chloroform-methanol brought about disintegration even of the formaldehyde fixed virus particles into granular masses.

The increase of buoyant density reflected the degree of delipidization and was in good agreement with the electron microscopic image. The higher the density the lower the proportion of particles with the typical ‘triple track’ membrane. For example after treatment with NP-40 and ether these particles accounted for about 20 to 30 % of the total number.
Fig. 1. Influenza virus PR-8 particles treated with caseinase C (60 µg) for 24 h. Negatively stained with silicotungstate. Apart from typical smooth spikeless particles (×) one can see a lot of broken particles displaying often a 'central body' separated from the remnants of virus membrane by a narrow space (arrows).

Fig. 2. Core-like structures prepared by treating formaldehyde-fixed spikeless particles with lysolecithin (0·5 mg/ml for 24 h) followed by saponin (10 mg/ml for 6 h). Negative staining with silicotungstate.

Fig. 3. Ultrathin section of 'saponin-cores' prepared by treating formaldehyde fixed spikeless particles with lysolecithin (0·5 mg/ml for 24 h) followed by saponin (10 mg/ml for 5 h). Note the amorphous material between the cores.
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Table 1. Effect of delipidization of spikeless PR-8 particles on their buoyant density in K-Na tartrate gradient

<table>
<thead>
<tr>
<th>Treatment applied to Fo-fixed spikeless PR-8*</th>
<th>Density of fractions containing subviral particles †</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td>1·130 1·130</td>
</tr>
<tr>
<td>2 Lyl (0·25 mg/ml, 35 °C, 18 h) + PL-A (2 mg/ml, 35 °C, 2 h)</td>
<td>1·100 1·120 1·160</td>
</tr>
<tr>
<td>3 NP-40 (0·2 %, RT°, 3 h)</td>
<td>1·135 1·175</td>
</tr>
<tr>
<td>4 NP-40 (0·2 %) + ether (4 °C, 4 h)</td>
<td>1·160 1·175</td>
</tr>
<tr>
<td>5 Lyl (0·5 mg/ml, 35 °C, 24 h) + NP-40 (0·2 %, RT°, 6 h)</td>
<td>1·160 1·175</td>
</tr>
<tr>
<td>6 PL-A (2 mg/ml, 35 °C, 24 h) + NP-40 (0·2 %, RT°, 4 h)</td>
<td>1·115 1·200</td>
</tr>
<tr>
<td>7 DOC (5 mg/ml, 35 °C, 30 min)</td>
<td>1·190 1·205</td>
</tr>
<tr>
<td>8 PL-A (2 mg/ml, 35 °C, 24 h) + ether (4 °C, 4 h)</td>
<td>1·190 1·215</td>
</tr>
<tr>
<td>9 DOC (10 mg/ml, 35 °C, 30 min)</td>
<td>1·135 1·210 1·240</td>
</tr>
<tr>
<td>10 Lyl (0·5 mg/ml, 35 °C, 24 h) + saponin (10 mg/ml, RT°, 6 h)</td>
<td>1·245</td>
</tr>
<tr>
<td>11 PL-A (2 mg/ml, 35 °C, 24 h) + saponin (10 mg/ml, RT°, 6 h)</td>
<td>1·250</td>
</tr>
</tbody>
</table>

* Fo, formaldehyde; Lyl, lysolecithin; PL-A, phospholipase-A; NP-40, Nonidet P-40; ether, diethylether 3: virus suspension (v/v); DOC, Na-deoxycholate; RT°, room temperature.
† Figures in italics indicate densities of the main bands.

whereas after saponin or DOC treatment they were practically absent. Therefore more detailed characterization was performed on materials obtained by means of saponin and DOC, respectively.

Cores prepared by treatment with saponin

Only one distinct band was obtained in tartrate density gradient after treating the spikeless particles with saponin for 4 to 6 h. Pre-treatment with lysolecithin or phospholipase A (Table 1) supported substantially the effect of saponin. Negative staining revealed well preserved round particles without any lipid membrane (Fig. 2). Ultrathin sections of this material showed that nearly all the particles were delineated by a single track shell but large aggregates of amorphous electron dense material were found in between the particles (Fig. 3). Thin-layer chromatography showed the presence, in reduced quantity, of all the lipid classes found under the same conditions in a control virus preparation: cholesterol ester, cholesterol, ceramide, cerebroside, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin (Fig. 4). A possible explanation would be that the amorphous material, trapped within the mass of core particles originated from the lipid bilayer. However, the interior of saponin cores was usually dark so that the internal structures were not clearly distinguished.

Cores prepared by treatment with DOC

DOC was used in final concentrations 0·5 or 1·0 % for 30 min at 36 °C without any marked difference. Usually only one distinct band was seen at a density over 1·20 g/ml, but when a higher concentration of virus suspension was used, additional faint bands could also be seen (Table 1). Electron microscopy of the materials of densities 1·20 and 1·24 revealed round granular bodies sometimes disintegrated. The granules measured about 4 nm when negatively stained with silicatungstastate (Fig. 5), but seemed to be condensed or aggregated.
Fig. 4. Thin-layer chromatography of chloroform-methanol extracts. The solvent mixture (1:1, v/v) was applied to the lyophilized specimens. Origin, double arrow. 1, control PR-8 virus (single arrow: spot of sucrose), 2, cores prepared by DOC treatment and washed twice before chromatography. 5, DOC extract of formaldehyde fixed spikeless particles. 4 and 3, first and second washings, respectively of DOC cores (arrow, large spot of DOC). A, cholesterol ester; B, cholesterol; C, ceramide; D, cerebroside; E, phosphatidylethanolamine; F, lecithin; G, sphingomyelin. Photograph is of two parts of the same plate.

into larger structures after negative staining with uranyl acetate (Fig. 6). Ultrathin sections showed round bodies with a single track 'membrane' on their surface (Fig. 7) and profiles similar to those of the nucleocapsid in the normal virus particles. However, broken particles were also present. Thin layer chromatography showed total absence of all the lipid classes typical for influenza virus although a threefold amount of virus material was used for the run. As expected they were identified in the DOC-extract (Fig. 4).

Polyacrylamide gel electrophoresis was used to characterize the polypeptide composition of the isolated 'central bodies'. The protein analysis of this material suffered from the use of fixed particles. In spite of the very strong dissociating treatment which was used before electrophoresis (boiling for 10 min with the SDS-urea-2-mercaptoethanol mixture), some
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Fig. 5. Formaldehyde-fixed spikeless particles treated with 1% DOC for 30 min at room temperature. Negative staining with silicotungstate before density gradient centrifugation. The cores are fairly granular, no bits of virus membrane can be seen.

Fig. 6. Same preparation as in Fig. 5, but negatively stained with uranyl acetate. Cores are more compact than after silicotungstate.

Fig. 7. Ultrathin section of 'DOC-cores' prepared as in Fig. 6 but banded in a tartrate density gradient.
material did not penetrate into the gel. The absence of P₁ and P₂ proteins which together account for a small proportion of the total protein content of the virion (Skehel & Schild, 1971) could possibly be explained in this way. Two bands were usually seen on gels, one in the position of the nucleoprotein at about 50,000 mol. wt. and another one moving rather faster than the M-protein. Subsequent studies have indicated that this was a cleavage product of caseinase C treatment of the M-protein. It has been shown that purified M-protein is sensitive to proteolysis by caseinase C and is converted into material of mol. wt. of about 15,000 (J. S. Oxford & B. Rentier, unpublished data). In addition, it has recently been found that caseinase C remains active even after formaldehyde fixation (M. Reginster, B. Rentier & L. Dierickx, unpublished data) and we did not remove caseinase C before processing the spikeless particles for delipidization. However, when caseinase C was washed out thoroughly a typical band of the M-protein (mol. wt. 25,500) appeared on the gel.

The antigenic properties of the nucleoprotein were preserved even after fixation with formaldehyde and treatment with DOC as detected by the single radial diffusion test and by complement fixation. On the other hand, the DOC-cores fixed complement in the presence of an antiserum against the M-protein (Oxford & Schild, 1975) but, using the same antiserum in the single radial diffusion test, the M-protein could not be detected in highly concentrated suspension of DOC-cores.

**DISCUSSION**

We have shown that it is possible to extract the lipids of influenza virus particles while preserving their internal organization if they have been fixed with formaldehyde. Thin-layer chromatography showed that the lipids were as efficiently extracted from the fixed as from the unfixed particles. This is in agreement with observations of Nir & Hall (1974) who were able to extract quantitatively all the lipids from formaldehyde fixed retina. On the other hand, glutaraldehyde prevents extraction of a significant proportion of lipids from brain tissue (Gigg & Payne, 1969) and retina (Nir & Hall, 1974). However, not only the typical structure of influenza virus interior was preserved but also the two main proteins (the nucleoprotein and the M-protein) were present and their antigenic reactivity was preserved. The main function of formaldehyde fixation seems to be cross-linking or stabilizing the surface shell which is presumed to be made up of the M-protein.

It seems that the virus core can exist without the lipid bilayer and that the 'core shell' may prevent entry of large molecules such as ribonuclease into the virus interior. It can also contribute to the rigidity of the virion together with the virus membrane. There is no doubt that the core shell is in close contact with the lipid bilayer in the native state and that the space seen after negative staining becomes larger during air-drying. On the other hand the stain must have penetrated this space before drying which suggests a hydrophilic interaction between the two structures. Using a lipid fluorescent probe (12-9-anthroyl-stearic acid) Lenard et al. (1974) demonstrated the transfer of energy from the M-protein to the probe located in the hydrophobic region of the lipid bilayer and calculated the maximum possible distance between the M-protein and the surface of the lipid bilayer to be 1.1 nm. Our observations of a maximum distance of 1 nm or less between those two structures are in good agreement with this value.

Several attempts have been made previously to obtain influenza virus cores. The DOC-cores described by Skehel (1971) were well characterized chemically by peptide analysis, RNA-analysis and specific density, but their internal organization was destroyed as demonstrated by ultrathin sections. Similarly, Schulze (1970, 1972) obtained 'cores' after glutaraldehyde fixation of influenza virus WSN and treatment with Nonidet, but no ultrathin
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sections were presented to show how the internal organization was preserved. Nermut (1972) was able to prepare cores of the strain X-3I fixed with glutaraldehyde and treated with phospholipase A, but in amounts insufficient for chemical characterization. Recently Dourmashkin & Tyrrell (1974) found virus cores in the cytoplasm of chorioallantoic membrane cells 2 h after infection as the first step of the uncoating process. This suggests that in a suitable environment the cores can exist without the lipid membranes.

Our results showed that the M-protein as well as the ‘single track’ structure remain in the cores, which were entirely devoid of lipids. Therefore we feel that it would be more appropriate to call the 25000 mol. wt. protein the ‘core protein’ instead of ‘membrane’ or ‘matrix’ protein as it is neither an intrinsic part of the virus membrane nor an amorphous pool of protein filling the internal space of the virion.

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