

Electron Microscopy of Influenza Virus

A Comparison of Negatively Stained and Ice-embedded Particles

F. P. Booy¹, R. W. H. Ruigrok^{2†} and E. F. J. van Bruggen¹

¹*Biochemisch Laboratorium, Rijksuniversiteit Groningen
Nijenborgh 16, 9747 AG Groningen, The Netherlands*

²*Biochemisch Laboratorium, Rijksuniversiteit Leiden
Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*

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An electron microscopical study was made of the influenza virus, type B/Hong Kong, in the unstained, frozen, hydrated state after quench-freezing in cooled liquid ethane. The results are compared with data from negatively stained specimens. It is shown that cryo-electron microscopy confirms and extends the data obtained by conventional methods. In particular, the virus is shown to be circular in projection with no indication of icosahedral symmetry, the lipid membrane is clearly resolved as a bi-layer and it is demonstrated that the distribution of material within the bi-layer is non-uniform, with a shell of more electron dense material surrounding a less dense central region. Neuraminidase spikes are not clearly distinguished from haemagglutinin spikes. The diameter of the complete B/Hong Kong virus was estimated from cryo-micrographs as 1270($\pm$ 70) Å. Some preliminary data for influenza virus type A/X31 are presented.

1. Introduction

The influenza virion has been studied extensively by electron microscopy using negative staining, freeze-fracturing, freeze-drying and conventional embedding and sectioning (see, for example, Wrigley, 1979; Nermut, 1972; Schulze, 1973).

Several models have been proposed for the structure of the virus. The validity of these models is frequently brought into question as a result of the many possible artefacts arising intrinsically from the conventional techniques of electron microscopy. So, there is still discussion about the precise shape and size of the virus, whether or not the coils seen within the virus are artefacts (see, for example, Fig. 5 of Schulze, 1972; Fig. 3(d) of Nermut & Frank, 1971), the origin of the so-called blebs (Nermut, 1972) and particularly about the precise arrangement of the M-protein and the ribonucleoprotein within the membrane.

Cryo-microscopy has become a more practical alternative and is expected to offer the possibility of examining isolated virus particles in a state much closer to that of their native environment (Lepault *et al.*, 1983; Adrian *et al.*, 1984). It is hoped that a

good cryo-electron micrograph effectively represents a snapshot of the viruses as they existed in aqueous suspension at the moment of freezing. There may, of course, still be some artefacts due to adsorption (to the carbon film or to the buffer-air interface) and due to freezing, but at least those artefacts due to staining and drying should be avoided.

We have applied the techniques of cryo-microscopy to influenza virus types A and B and report here how the results compare with negative staining experiments and other data in the literature.

2. Materials and Methods

Influenza virus preparations were generously provided by Duphar (Weesp, the Netherlands) and the RIVM (Bilthoven, the Netherlands). Strains B/Hong Kong/8/73 and A/X31 (H3N2 recombinant strain of A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2)) were investigated. The B/HK strain had been inactivated with 0.01% (w/v) *b*-propiolactone and purified to monodispersity as described (Mellema *et al.*, 1981). The X31 was neither inactivated nor purified to monodispersity. Both strains were in PBS (140 mM-NaCl, 3 mM-KCl, 10 mM-phosphate buffer, pH 7.0), with 0.01% (w/v) sodium azide.

Negatively stained preparations were made by adsorbing the virus onto freshly prepared carbon film

† Present address: MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, Great Britain.

deposited on mica. The film was subsequently floated onto 0.1 M-ammonium acetate solution at pH 7 to wash away any non-volatile salts and stained with phosphotungstic acid (buffered at pH 7) or uranyl acetate (unbuffered). These preparations were examined in a Philips EM 300 electron microscope.

Cryo-microscopy was carried out using a Philips EM 400T microscope equipped with a cooling holder (type PW 6591) and a blade-type anti-contamination device. A drop of the virus suspension was placed on a grid coated with either a carbon or a holey carbon supporting film, allowed to adsorb for some 30 s, blotted partially dry with filter paper and quench-frozen in liquid ethane maintained close to its freezing-point by liquid nitrogen. A home-made guillotine-like device was used for freezing. The frozen grid was then transferred to the cooling holder under liquid nitrogen, inserted into the microscope and examined at approx. -160°C . Micrographs were recorded after about 15 min with a low level of liquid nitrogen in the cooling holder, which was used as described by Booy & van Bruggen (1984). The minimum dose required to provide a printable optical density on Kodak 4463 films was used. At $20,000\times$ magnification this was some 2 to $5\text{ e}/\text{\AA}^2$, resulting in an optical density of approx. 0.5. Areas of virus in a thin film of amorphous buffer were located in the diffraction mode with the diffraction lens overfocused to provide an out-of-focus and thus high-contrast image at a very low magnification. Using the low-dose unit, the beam was blanked and the M button pressed to give a preset imaging condition without any further adjustment. Focusing was carried out in the deflected position and micrographs were recorded in the range 0 to $15\text{ }\mu\text{m}$ underfocus.

3. Results

Figure 1 gives the general appearance of the B/Hong Kong virus in the unstained, frozen state at low magnification. A large number of circular particles is seen with a layer of spikes clearly visible around the circumference. The spikes are further revealed as a stippling over the surface. There are still some misshapen, broken and outsize particles present but the majority have a well-defined circular shape and are uniform in size. There are no flattened sides visible to indicate icosahedral symmetry.

Figure 2 shows a similar preparation at a higher magnification on a holey carbon film. (The hole size is several μm and extends across almost the entire field of view.) Arrows draw attention to small particles, uniform in size, that are occasionally seen in close proximity to virus particles of normal size.

Figures 3 to 5 illustrate the influence of varying the focus on the appearance of the virus. Figure 3 is very close to focus and the spikes can no longer be resolved individually. Occasionally, a darker ring is seen within the virus (arrow). Figure 4 is slightly underfocused (approx. 2 to $4\text{ }\mu\text{m}$). It is again possible to resolve individual spikes and the lipid membrane is now clearly revealed as a distinct bi-layer with a spacing of about $40\text{ \AA}$ (arrows). Figure 5 is still further underfocused (approx. $10\text{ }\mu\text{m}$) and the visibility of the lipid bi-layer is lost, whilst the contrast of the spike proteins is enhanced. A

comparison with an unpurified A/X31 preparation is shown in Figure 5. It is clear that the preparation has been very much less purified. There are many free spikes in the background (seen as highly contrasted dots) and empty and elongated particles. Sometimes, twisted ribbon-like structures are seen that we consider to be the ribonucleoprotein material from disrupted viruses. The inset shows an interesting structure within a virus that was seen on one occasion.

Figures 6 and 7 show the B/Hong Kong preparation negatively stained with uranyl acetate and phosphotungstic acid, respectively. In Figure 6, arrows point to virions in which the negative stain has penetrated the lipid layer and contrasted the internal components of the virus.

From micrographs such as Figure 2, the diameter of the complete virus particle was estimated; firstly, using a Hewlett Packard 8964A digitizer coupled to a 9820A calculator and, secondly, using a Zeiss TGZ3 particle size analyser. The diameter of the B/HK virus was found to be $1270(\pm 70)\text{ \AA}$. From micrographs such as Figure 4, in which the lipid membrane is clearly resolved, the diameter of the virus to the centre of the lipid membrane was estimated and thus by subtraction the spike length protruding from the outside of the lipid membrane was estimated as $137(\pm 7)\text{ \AA}$. The diameter of the lipid bi-layer itself was directly estimated from cryo-micrographs as $40(\pm 5)\text{ \AA}$.

4. Discussion

The results presented show that cryo-microscopy will indeed be most helpful in the investigation of the structure of viral particles. So, in Figure 1 it is clear that the shape and size of the virus particles have been well-preserved when compared with similar negatively stained preparations (Figs 6 and 7). Phosphotungstic acid, whilst clearly contrasting the spike proteins around the virus, also distorts the shape somewhat, often resulting in a kidney-shaped virus. Uranyl acetate, on the other hand, preserves the shape rather better and, whilst contrasting the spikes over the surface of the virion well, does not reveal them around the edge so clearly. In reality, it is surprising just how reliable negative staining appears to be. Neither negative staining nor cryo-microscopy reveals the regular arrangement of spikes over the surface of the virion that was seen for the icosahedral Semliki Forest virus (Adrian *et al.*, 1984). Furthermore, neither technique clearly demonstrates more than one type of spike protein, even though after isolation and purification the spikes can be distinguished (Laver & Valentine, 1969). This is probably due to the tight packing and superposition of the spikes over the virion, which confuses the substructural detail. The small particles (the so-called blebs) that are often seen in association with stained particles (for example in Fig. 6 here) are also present in the cryo-pictures (Fig. 2) and are thus almost certainly

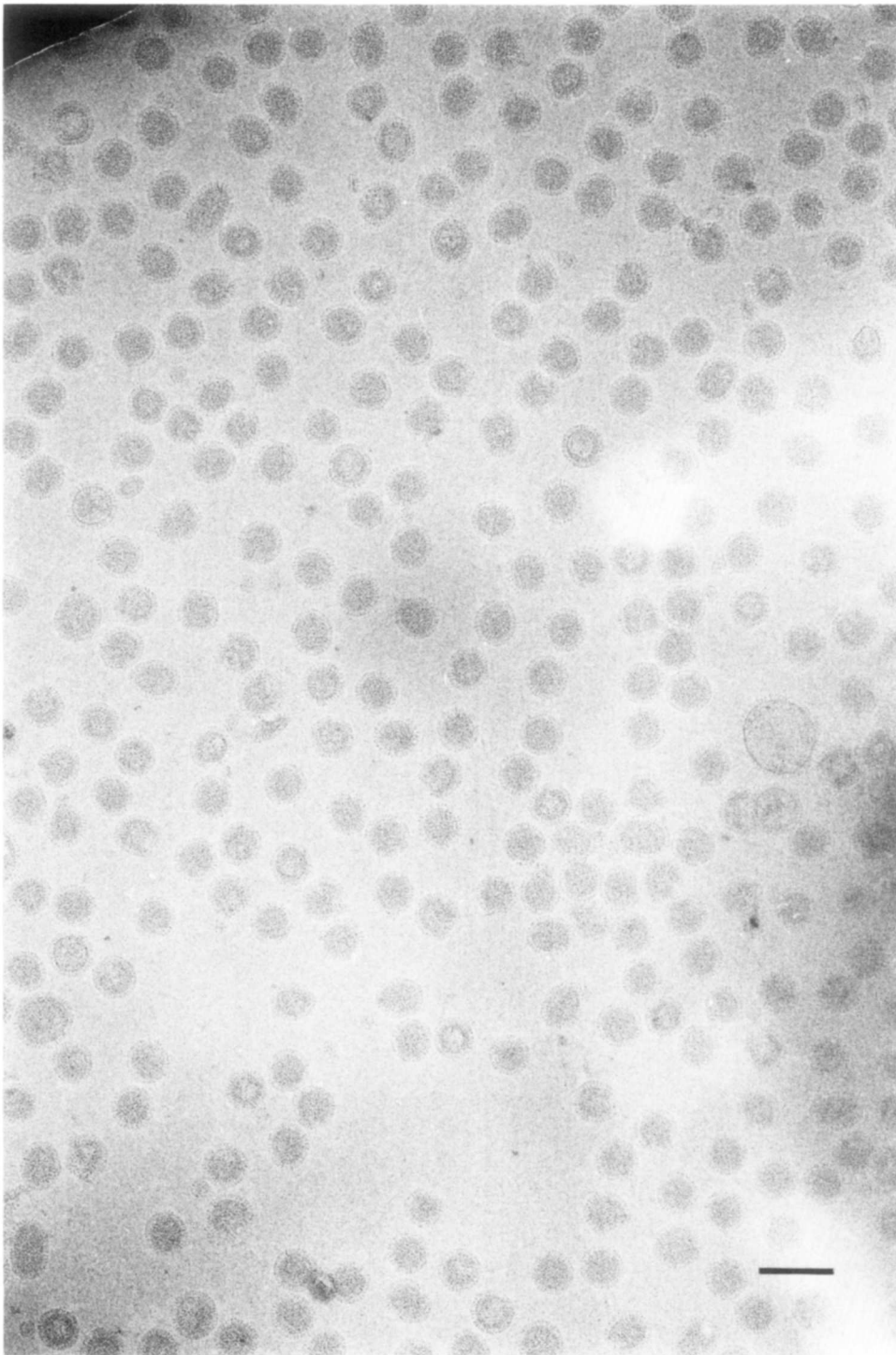


Figure 1. Electron micrograph of an unstained, frozen, hydrated specimen of B/HK influenza virus prepared on a carbon supporting film. The bar represents 2000 Å.

storage artefacts rather than staining artefacts. There is, however, an additional important difference between a micrograph of a negatively stained virion and a micrograph of an unstained, frozen, hydrated virion that provides significant extra data: it is that in the stained case the contrast is due to scattering from the heavy-metal atoms that are mapping out the surface features of the

virion. Unless the stain penetrates the viral envelope (causing disruption of the structure), there is effectively no contrast from internal components, even though it is a transmission micrograph. For the unstained, frozen virion the matter is quite different and rather more complicated. Firstly, contrast arises as a result of differences in scattering power of the surrounding buffer, the spikes, the

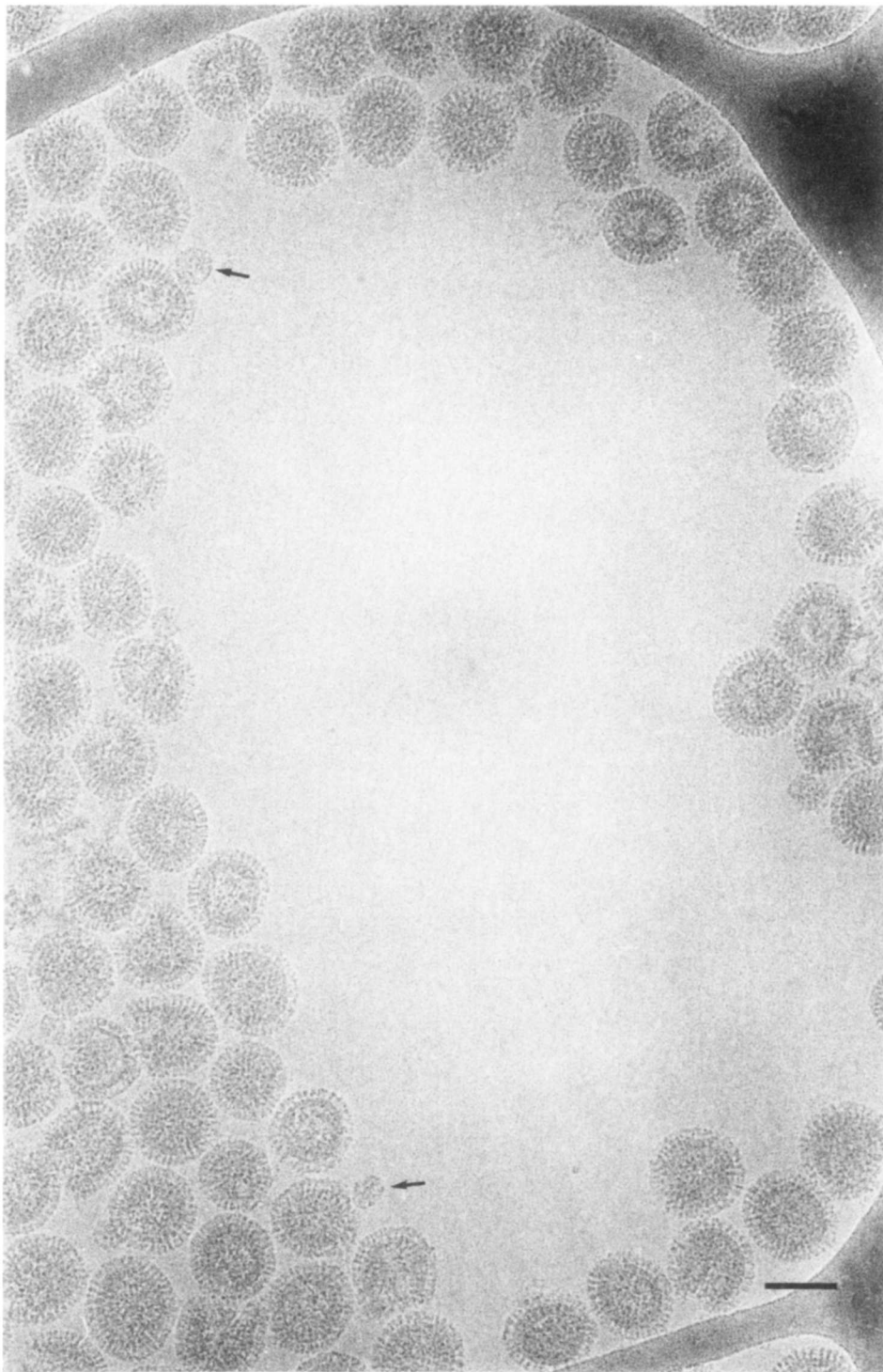


Figure 2. Well-underfocused electron micrograph of an unstained, frozen, hydrated specimen of B/HK influenza virus prepared on a holey carbon supporting film. For arrows, see the text. The bar represents 1000 Å.

membrane and the internal components. Thus, the information from the upper and lower surfaces of the virus is superimposed on the information from the internal components. The picture is then further complicated by the fact that phase contrast plays a very much more significant role in these unstained preparations, as is well-illustrated by Figures 2 to 4. Much further work is required before a complete

interpretation of such cryo-micrographs is possible. Close to focus, the microscope has a transfer function that (to a first approximation) transmits a uniform, broad spectrum of frequencies and phase contrast makes a negligible contribution. To the eye, such a micrograph shows very little contrast. As the objective lens is defocused, the contrast transfer function enhances different spatial

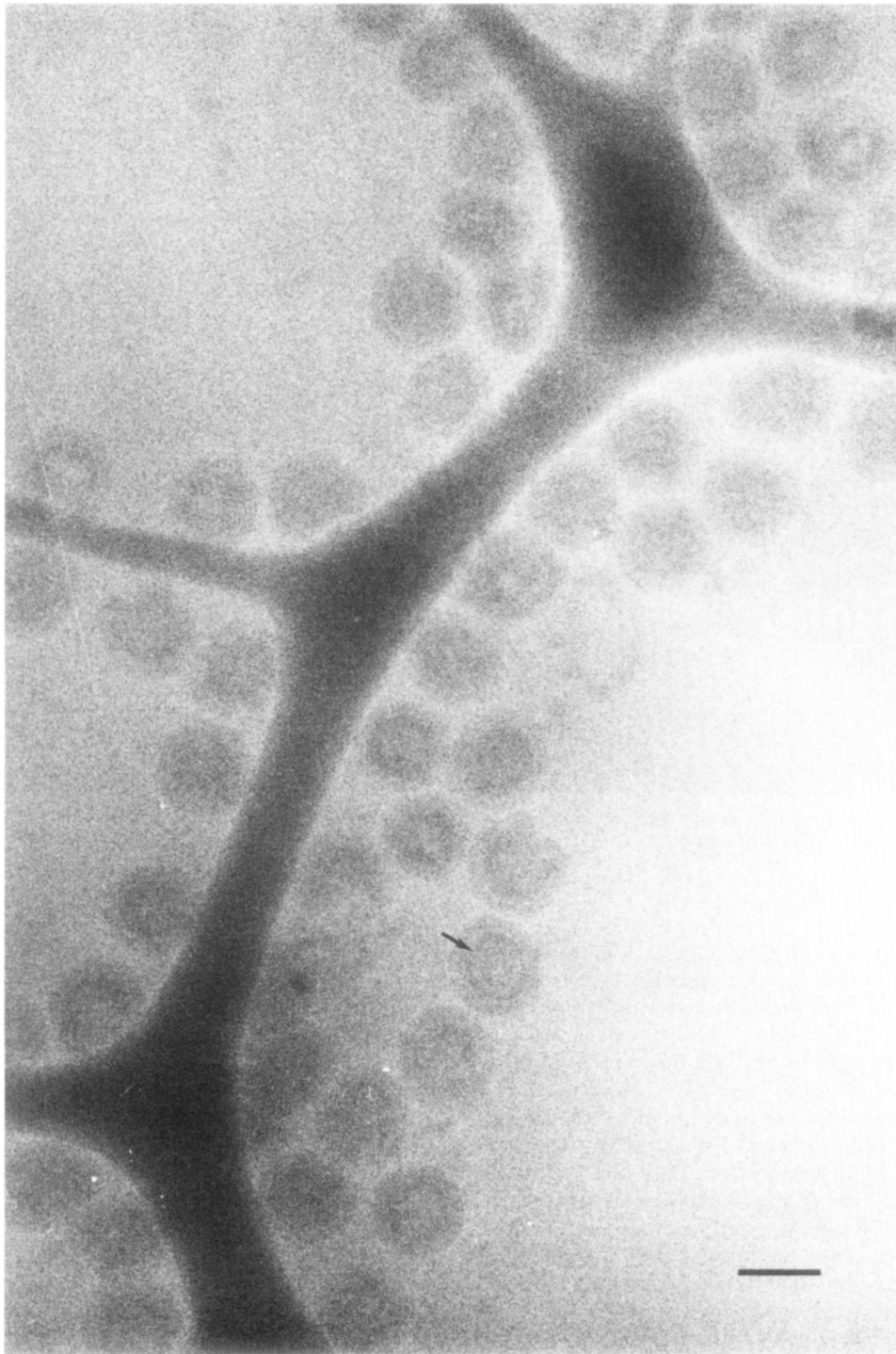


Figure 3. Electron micrograph of a specimen as in Fig. 2 but photographed close to focus. The arrow indicates a more electron dense ring within the bi-layer. The bar represents 1000 Å.

frequencies and, by eye, the micrograph shows different features. So, in Figure 4, slightly underfocus, the contrast of the lipid membrane is enhanced and in Figures 2 and 5, further underfocus, the contrast of the spike proteins is enhanced. Unfortunately, no defocus value enhances the visibility of the internal components of the virus alone. However, their scattering

contributes information in all micrographs. Most noticeable is the dark ring within the viral membrane that is clear in many of the virions in Figure 3. Although the eye detects this ring most easily in Figure 3 when the contrast of the spikes and lipid is not enhanced, a similar dark band is also visible in Figures 1 to 4. In fact, this is really quite surprising, since in a transmission micrograph

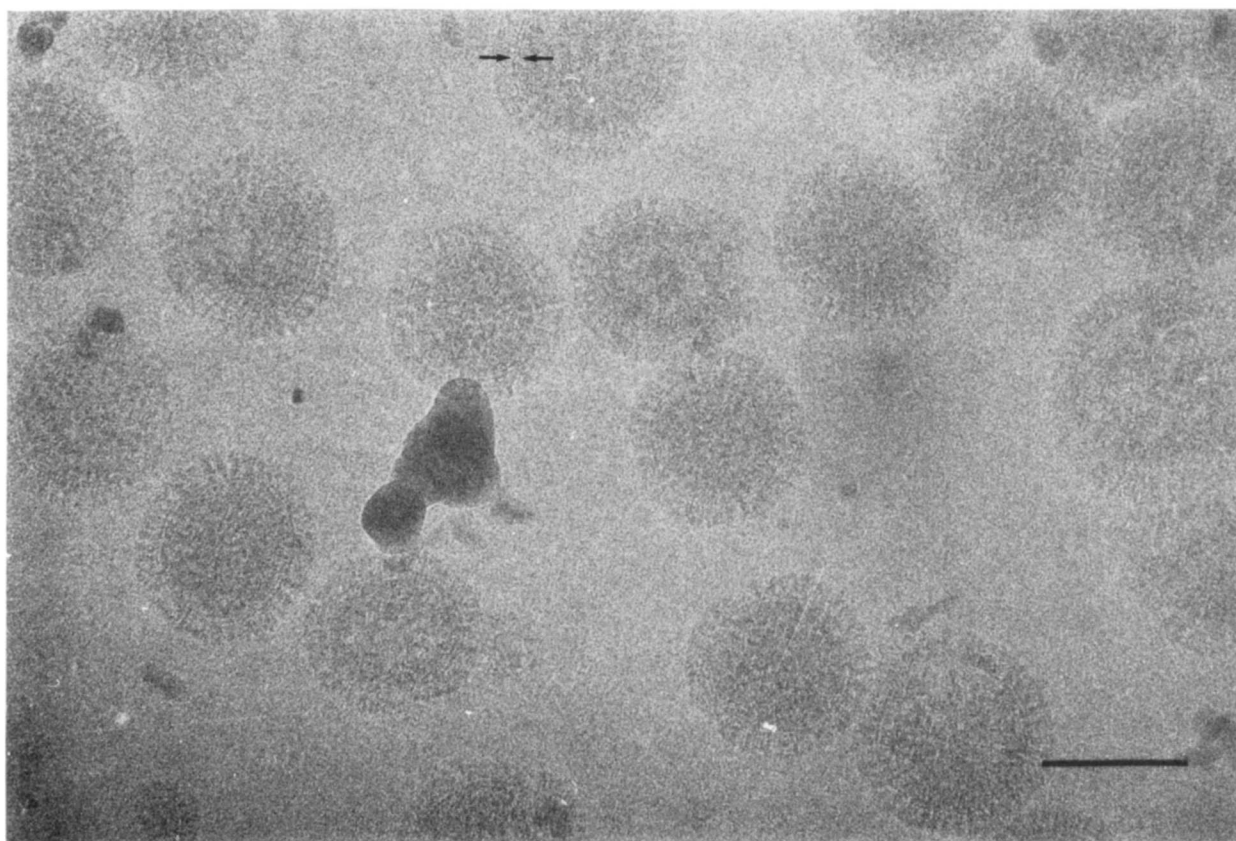


Figure 4. Electron micrograph of a specimen as in Fig. 2 but photographed just under focus. The arrows indicate the lipid bi-layer. The bar represents 1000 Å.

it would be expected that a more or less uniformly filled sphere would become progressively darker towards the centre of the sphere. In addition to the dark ring mentioned above, many virus particles actually become lighter towards the centre. It must therefore be concluded that the interior of the virion is far from uniform, with much of the denser and thus more strongly scattering material residing away from the centre. This finding rather nicely confirms the early work involving freeze-fracturing and sectioning (see, for example, Schulze, 1973), in which it was postulated that the centre of the virus was mostly devoid of protein and RNA. It is also further confirmed by negative staining experiments, in which the stain penetrates the lipid membrane (arrows, Fig. 6) leaving a ring of stain-excluding material within the lipid membrane, which compares with the electron-dense ring noted in Figures 1 to 4.

Figure 5 is a rather interesting micrograph. The unpurified A/X31 virus is revealed as being much less uniform in both size and shape. Many of the particles are partly or even totally empty and/or devoid of spikes. Sometimes the spikes appear to be present only around the circumference of the virus. In the background, many free protein spikes can be seen in end-on view, presumably with their hydrophobic ends at the top or bottom of the ice-layer. At the limited resolution of the micrograph,

neither trimers of haemagglutinin nor tetramers of neuraminidase can be distinguished readily.

A number of twisted or helical ribbons is also to be seen in the background and this is thought to be the ribonucleoprotein material from disrupted virions (compare with Fig. 5 of Jennings *et al.*, 1983). Sometimes there is an indication of these ribbons being visible within a virus, presumably in just the appropriate orientation (arrows, Fig. 5). On one occasion a virus containing a very much larger structure (Fig. 5, inset) was found. Measurements indicate that this is probably an example of a coiled structure similar to those previously seen in negatively stained preparations of disrupted viruses (see, for example, Fig. 3(d) of Nermut & Frank, 1971).

Figures 1 to 5 demonstrate also that the virus particles are not equivalent in all details. The presence of a more electron dense ring of material seen in some B/HK particles suggests that not all the viral particles have the full complement of internal core components. This may help explain the larger than expected standard deviation in scanning transmission electron microscopic mass determination experiments on these particles (Ruigrok *et al.*, 1984) and supports the interpretation of neutron scattering data (Cusack *et al.*, 1985). It is further implied that more than one viral particle is essential for viral expression.

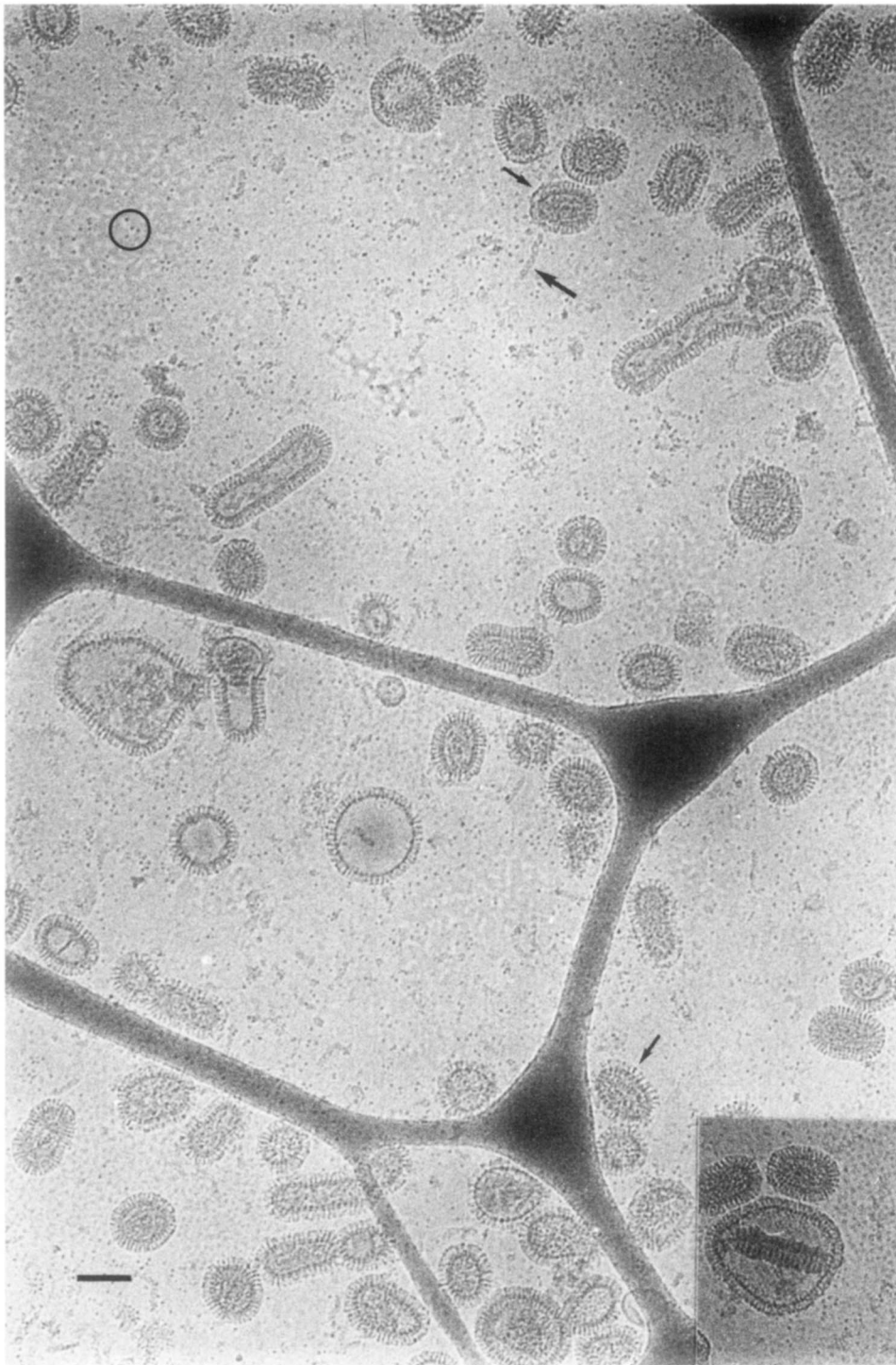


Figure 5. Well underfocused electron micrograph of an unstained, frozen, hydrated specimen of A/X31 influenza virus. Some free haemagglutinin spikes are ringed. The arrows point to free ribonucleoprotein material from disrupted virions and also to virions in which an indication of the ribonucleoprotein is seen. The inset shows an example of a large coiled structure within a virion. The bar represents 1000 Å.

The diameter of $1270(\pm 70)$ Å for B/HK virus particles determined from the cryo-electron micrographs agrees well with the hydrodynamic radius of $635(\pm 4)$ Å (Ruigrok, 1985). There was no measurable size difference when the sodium chloride in the buffer was exchanged for potassium chloride, as

was predicted by hydrodynamic measurements. The measured size is somewhat larger than the 1190 Å predicted from neutron scattering studies (Cusack *et al.*, 1985) and the 1206 Å estimated from negatively stained preparations (Ruigrok, 1985).

Cryo-micrographs of specimens tilted to 40° gave

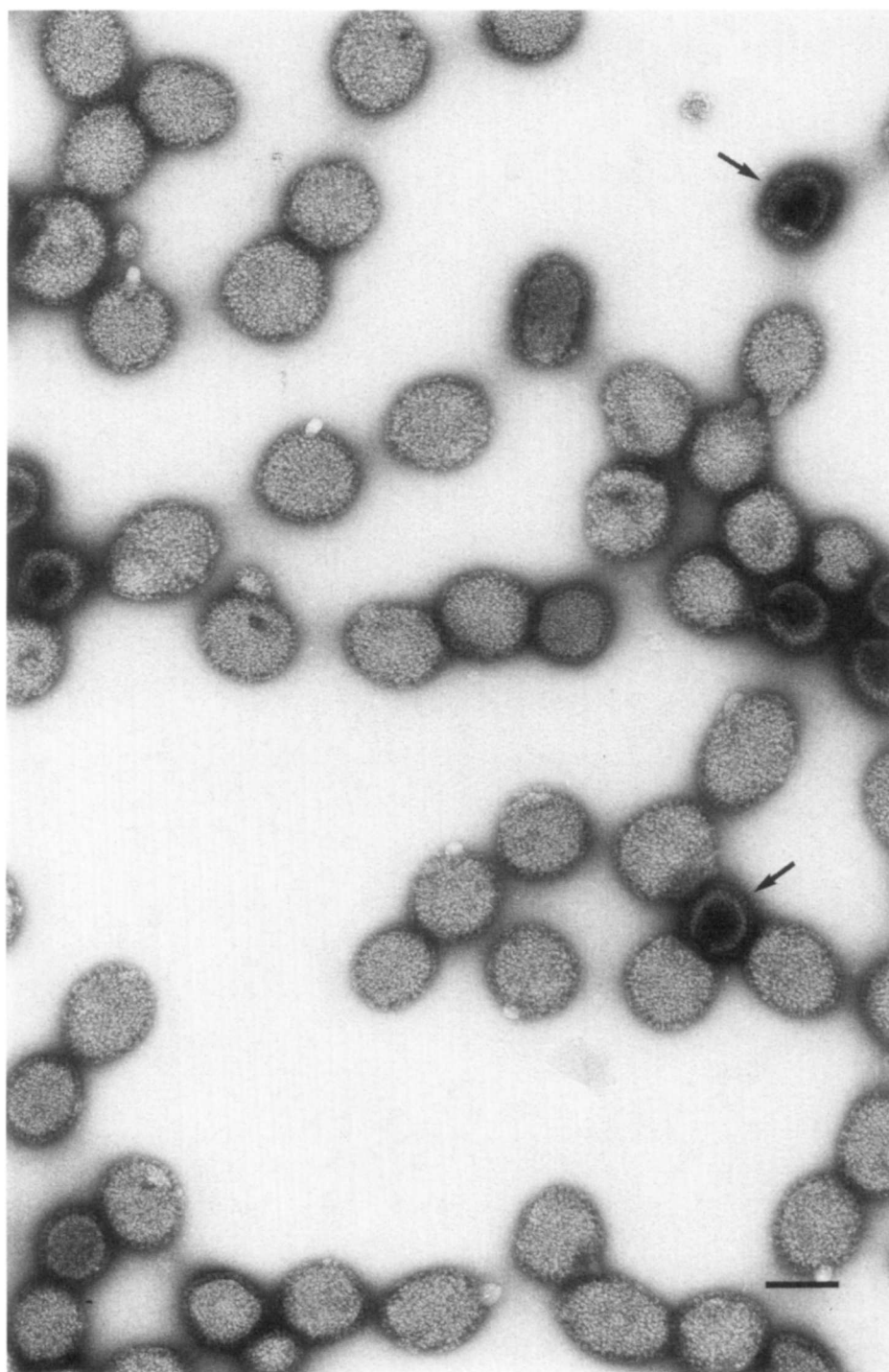


Figure 6. Electron micrograph of a specimen of B/HK influenza virus negatively stained with uranyl acetate. Arrows indicate virus particles in which the stain has penetrated the lipid membrane and contrasted the internal components of the virus. The bar represents 1000 Å.

no indication of flattening due to freezing in a thin film of buffer and, according to Adrian *et al.* (1984), a linear expansion of less than 2% is to be expected as a result of vitrification. All of these hydrodynamic, neutron and electron microscopical measurements were carried out using the same peak fraction of the virus preparation. The estimated spike length of 137 Å protruding from the lipid

bi-layer in cryo-electron micrographs agrees well with the data derived from a crystallographic study of bromelain-released haemagglutinin (Wilson *et al.*, 1981).

In virus particles such as those shown in Figure 2, the number of peripheral spikes seen in projection is $38(\pm 2)$. With the assumption that only a single layer of spikes is counted and that the

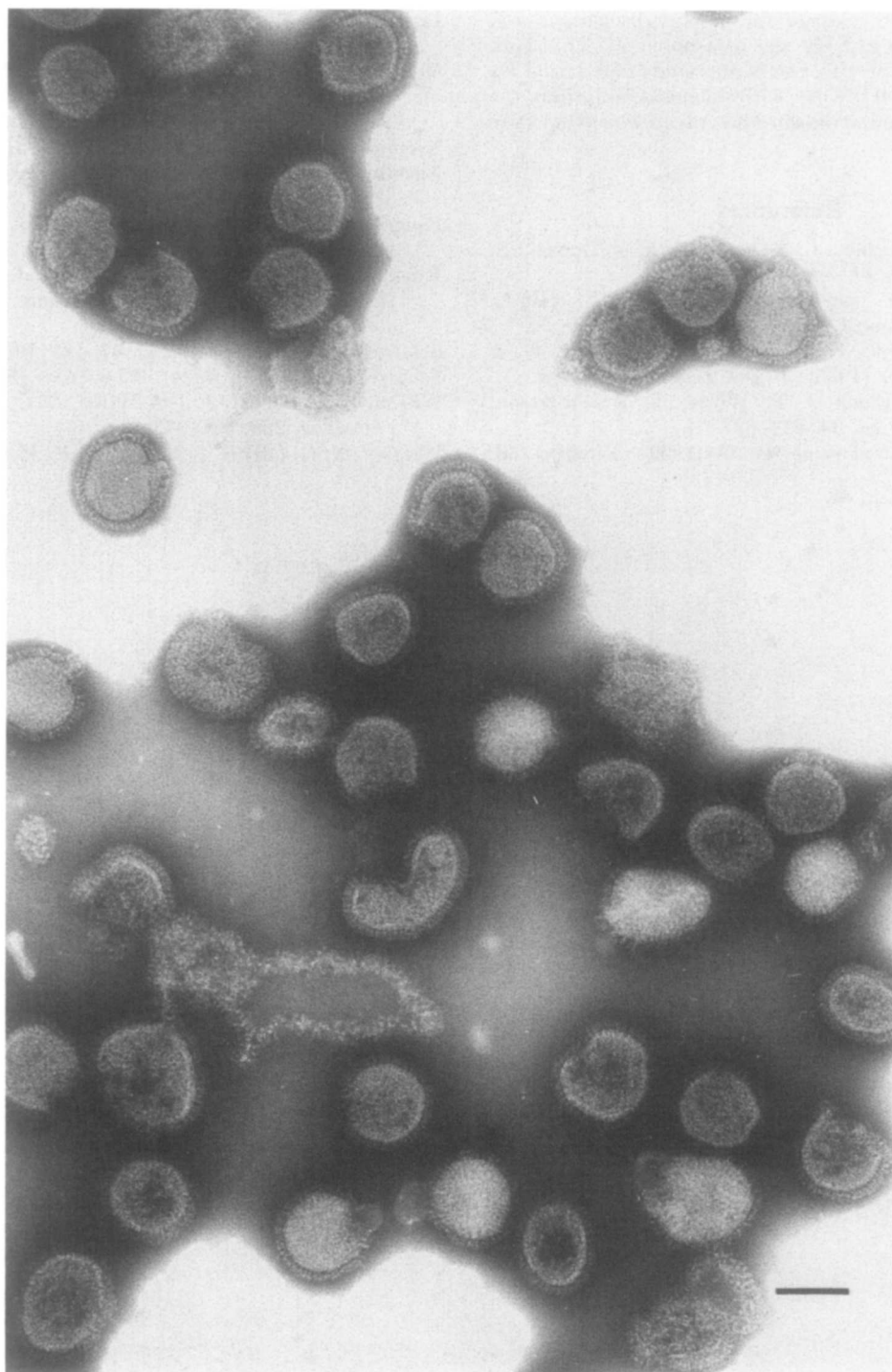


Figure 7. Electron micrograph of a specimen of B/HK influenza virus negatively stained with phosphotungstic acid. The bar represents 1000 Å.

difference between neuraminidase and haemagglutinin spikes is negligible, this would be expected to give rise to a maximum of some 530 spikes over the complete virus particle (assuming a close-packed structure for the spikes).

5. Conclusions

Cryo-electron microscopy confirms that B/HK influenza virus is spherical with an overall diameter

of $1270(\pm 70)$ Å. It is studded with spikes that protrude some 137 Å from a clearly resolved lipid bi-layer. Two types of spike protein are not clearly distinguished. Within the lipid membrane, the distribution of ribonucleoprotein is non-uniform, with the bulk of the more electron dense material residing away from the centre.

In general, the published data from negative staining, freeze-fracturing and sectioning studies are confirmed and extended by the technique of cryo-microscopy alone.

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