

## Studies on the Structure of a Coronavirus-Avian Infectious Bronchitis Virus

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### SUMMARY

When avian infectious bronchitis virus (IBV) is fixed in formaldehyde, negative stain is able to penetrate the particle and an internal component is visualized. This component is seen as a tongue or flask shaped structure attached at one point to the outer virus membrane. A model yielding transmission patterns similar to the virus has been made. Gradient centrifugation studies on IBV reveal that the RNP is associated with the internal sac.

### INTRODUCTION

The coronaviruses are recognized by their ether lability, apparent RNA content and unique morphology (Tyrrell *et al.* 1975). By electron microscopy they are 80 to 160 nm in diam. and have characteristic club shaped surface projections 10 to 11 nm in length. Prepared by the thin sectioning technique, coronaviruses generally appear rounded (Becker *et al.* 1967; Nazerian & Cunningham, 1968; Apostolov, Flewett & Kendal, 1970; Uppal & Chu, 1970; Cowen, Hitchner & Ubertaini, 1971), but by negative staining they are rather more irregular in outline (Berry *et al.* 1964; Estola & Weckstrom, 1967; McIntosh *et al.* 1967). No interior ribonucleoprotein strand has been seen in any negatively stained preparations, although there has been one report of a helical component released from virions of the human coronavirus, strain 229E (Kennedy & Johnson-Lussenberg, 1976). Occasionally electron-dense structures in the centre of virus particles have been observed in ultrathin sections (Apostolov *et al.* 1970; S. Patterson & R. W. Bingham, unpublished results), but it is not known whether these represent ribonucleoproteins. Another characteristic feature of the virion in ultrathin section is the presence of a distinct pair of electron dense shells. In the case of avian infectious bronchitis virus (IBV), Becker *et al.* (1967), Nazerian & Cunningham (1968) and Cowen *et al.* (1971) showed that the inner shell was in close apposition to the outer, whereas Uppal & Chu (1970) showed the presence of an electron-lucent space between the two. This electron-lucent space has also been observed in sections of other coronaviruses - mouse hepatitis virus (David-Ferreira & Manaker, 1965) and the human strain, 229E (Becker *et al.* 1967).

Recently Garwes, Pocock & Pike (1976) have shown that in the case of the porcine coronavirus, transmissible gastroenteritis virus (TGEV), detergent treatment of the virus removes the external lipoprotein membrane and projections, leaving smaller approximately spherical particles of diam. 60 to 70 nm. It is suggested that these subviral particles represent the inner shell observed in ultrathin sections.

Further evidence for an inner membrane comes from an experiment by Berry & Almeida

(1968). They showed that by using antibody and complement the outer membrane of IBV was rendered permeable to phosphotungstate negative stain. Under these conditions, an unusual tongue shaped membranous sac could be clearly seen inside the virion (Fig. 1).

In this report we describe further studies on the internal morphology of IBV.

#### METHODS

*Virus.* The strains of IBV used, and their method of growth *in ovo* have been described previously (Bingham, 1975).

*Purification and treatments applied to virus.* IBV was purified from allantoic fluid as described previously (Bingham, 1975), with the exception that the phosphate buffered saline used formerly was replaced by 0.05 M-2(*N*-morpholino)ethane sulphonic acid (MES) adjusted to pH 6.5 with KOH. Direct electron microscopy experiments were usually carried out after the first sucrose gradient without further virus purification. Isolation of fractions of detergent treated virus was attempted as follows. Sucrose gradient purified virus was diluted in MES buffer, pelleted at 78000 *g* for 1 h and resuspended in 0.5 ml 5% formaldehyde. This was layered over 2 ml of 10% (w/w) sucrose containing 0.5% Nonidet P40 (NP40) which, in turn, was on top of a 10 ml linear gradient of 25 to 70% (w/w) sucrose. This gradient was centrifuged at 110000 *g* for 16 h at 4 °C and finally collected in 1 ml fractions. Extinction at 280 and 260 nm and the refractive index were measured for each fraction. The RNA and protein content of each fraction were estimated from the  $E_{260}$  and  $E_{280}$  values by the method of Warburg & Christian (1941).

*Electron microscopy.* Negative staining was carried out by placing drops of the virus-containing fractions directly on to 400 mesh grids coated with carbon-formvar. After allowing a few seconds for virus adsorption excess fluid was withdrawn with filter paper and the grid washed carefully with drops of distilled water, which were then replaced with drops of either 1.5% phosphotungstic acid adjusted to pH 6.5 with KOH or 1.5% molybdophosphoric acid adjusted to pH 6.5 with  $\text{NH}_4\text{OH}$ . Excess stain was then withdrawn with filter paper and the grid examined in either a Philips EM300 or an AEI EM6B electron microscope at an accelerating voltage of 80 kV. In some experiments, before negative staining was carried out, the virus suspension was fixed by the addition of an equal quantity of either 10% (w/v) formaldehyde solution or 5% glutaraldehyde.

In addition, some of the virus preparations were degraded with the detergent NP40 on the grid. This was carried out by allowing the virus to adsorb to the grid as before then washing very briefly (2 to 3 s) with 0.5% NP40. Immediately the detergent was replaced with distilled water and negative staining carried out as before (Almeida & Brand, 1975).

#### RESULTS

Brief exposure of the virus to the detergent NP40 directly on electron microscope grids resulted in a rapid breakdown of the virus envelope, allowing penetration of the negative stain into the interior of the particles. This revealed an inner membranous sac (Fig. 2) similar to those described by Berry & Almeida (1968) for antibody and complement treated virions. This inner membrane always appeared to be attached to the outer envelope at one point, and in many particles an orifice could be seen at this point of attachment (Fig. 2). If the exposure to detergent was for longer than about 15 s, the outer envelope of the virus was partially or completely destroyed but many of the inner sacs were left intact. Even longer exposure to the detergent resulted in the destruction of these also. It would appear that the internal structures visualized are not artifacts resulting from the detergent treatment, as

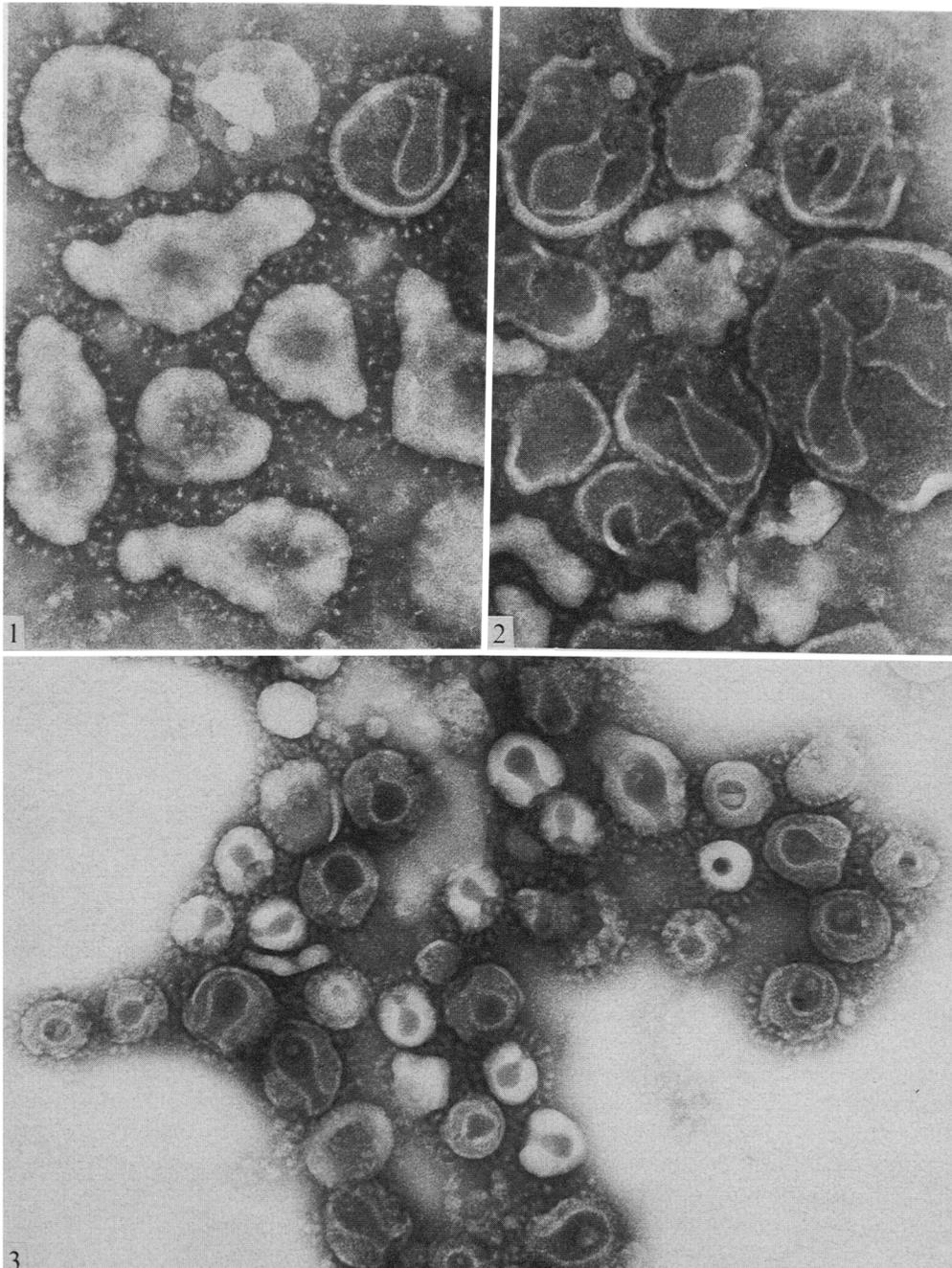


Fig. 1. A group of avian infectious bronchitis (IBV) virus particles. They display the pleomorphic appearance and distinctive projections characteristic of the coronavirus group. The particle at the upper right of the micrograph has been penetrated by stain and within it can be seen a tongue shaped structure. Magnification  $\times 150000$ .

Fig. 2. IBV particles, treated on the grid with non-ionic detergent, quickly lose the surface component of the virus. The stain is allowed to penetrate and the internal tongue shaped structures can once again be seen. Magnification  $\times 150000$ .

Fig. 3. A group of IBV virions treated with formaldehyde before staining with phosphotungstic acid. Almost all now reveal the internal component. The majority display the tongue or flask orientation but others show a circular structure or even two concentric rings. Fig. 7 shows that all of these patterns are compatible with an internal membranous sac continuous with the outer membrane. Magnification  $\times 135000$ .

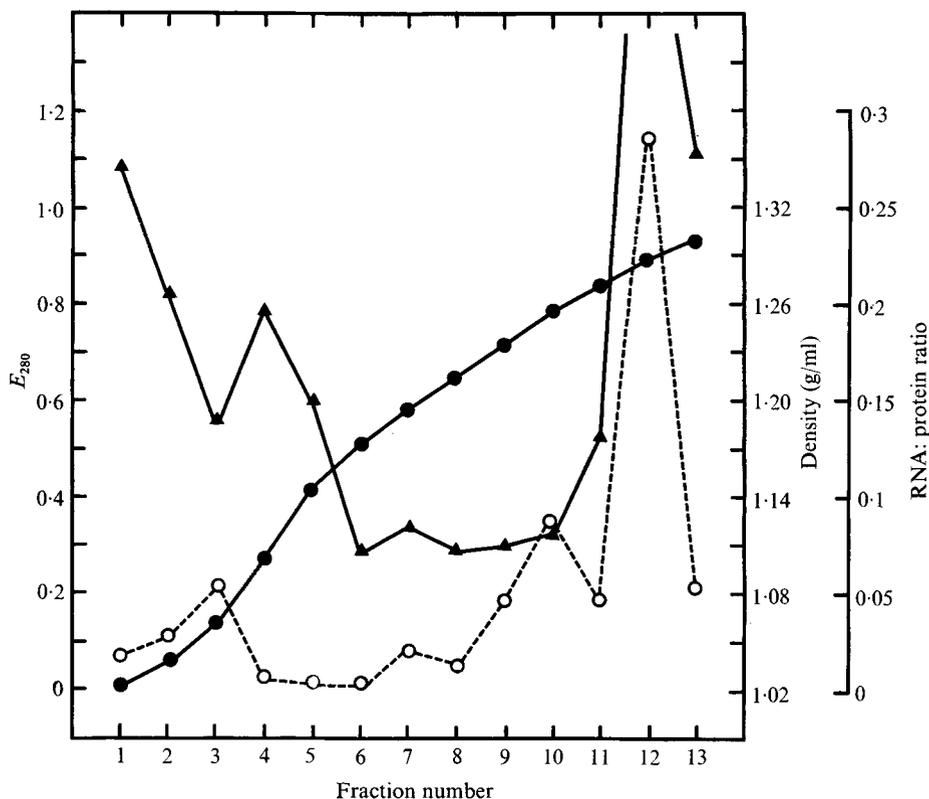


Fig. 4. Distribution of RNA and protein after sucrose gradient equilibrium centrifugation of detergent treated IBV. ●—●, Density g/ml; calculated from its refractive index at 20 °C. ▲—▲,  $E_{280}$ ; the extinction at 280 nm of fraction 12 was too large for direct measurement. ○---○, RNA: protein ratio, determined from diluted samples where appropriate.

they are also occasionally observed in untreated virus preparations (Fig. 1). The inner components varied in shape from long tongue-like forms to nearly spherical sacs, but they usually featured a constricted opening at the point of attachment to the outer envelope. Although numerous preparations were examined, no helical or other recognizable ribonucleoprotein component was ever observed.

In an attempt to preserve any internal structure that may have been too labile to be observed in the usual virus preparations, particles were fixed with either glutaraldehyde or formaldehyde. The glutaraldehyde treated preparations were indistinguishable from the untreated, apart from an increased aggregation of the particles. However, formaldehyde fixed particles showed some interesting features. It appeared that treatment with this fixative allowed penetration of the outer virus membrane by the negative stain and the majority of the particles showed the inner membrane, again arranged in the form of an open flask with the opening attached to the virus envelope (Fig. 3) and apparently continuous with it. In other particles the orientation was such that the entrance to the inner compartment was perpendicular to the beam. Some particles did not show a clearly defined membrane, but possessed a diffuse circular internal structure (top right of Fig. 3).

In an attempt to isolate the inner component, formaldehyde fixed virus was sedimented through a layer of NP40 into a 25 to 70% (w/w) sucrose density gradient. A distinct band

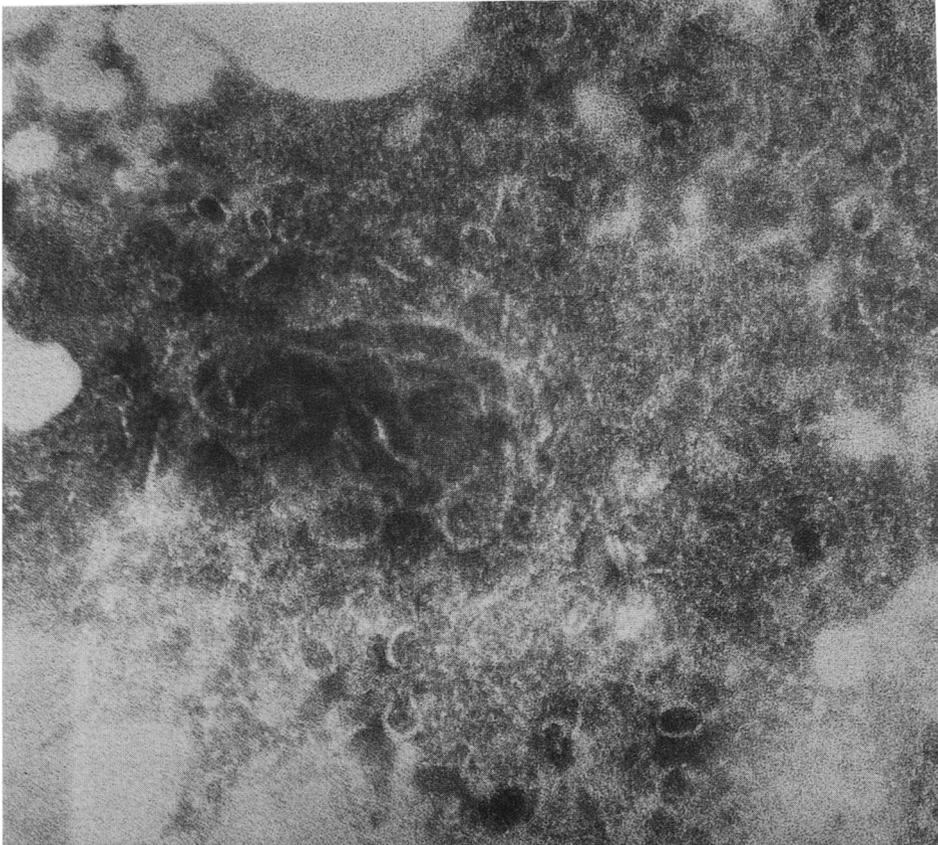


Fig. 5. Electron micrograph of material found in fraction 12 of the gradient depicted in Fig. 4. No recognizable particles are present but membranous structures compatible with the inner sac are seen. Magnification  $\times 130000$ .

of material was observed after the gradient had been centrifuged overnight. The extinctions at 280 and 260 nm, and refractive indices of the 1 ml fractions collected were measured (Fig. 4). The visible band was in fraction 12 and had a density of 1.27 to 1.29 g/ml in two experiments. When this material was examined in the electron microscope (Fig. 5) only aggregates of membrane-like structures, which could be remnants of the inner membranes described above, were seen. The RNA:protein ratio of this fraction was 0.284 compared with a value of 0.069 for whole virus (Bingham, 1975). However, the electron microscope appearance was more characteristic of membranous material than strands or filaments of a ribonucleoprotein.

#### DISCUSSION

To facilitate understanding of the internal component of the virus we have constructed a model to correspond with the forms observed (Fig. 6). This model was formed from glass so that it could be photographed by transmitted light in various orientations. Photographs so produced showed a close similarity to the different electron microscope images obtained (Fig. 7). We believe that the observed structure of the virus consists of an outer lipid envelope, bearing the characteristic peplomers, and an inner membranous 'flask' which is attached to the outer membrane by its neck. In the natural state the outer envelope is

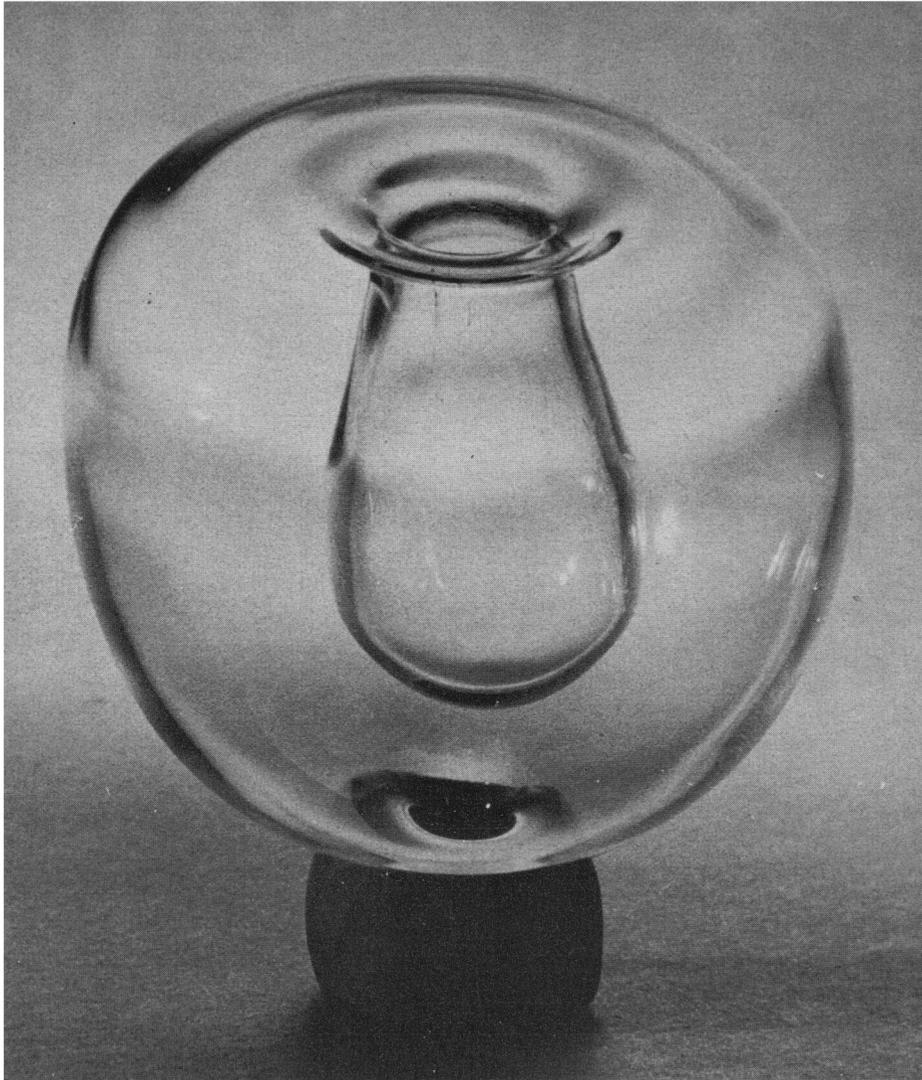


Fig. 6. A glass model consisting of an inner tongue and a continuous outer sphere. Viewed by back illumination the model yielded patterns that could be compared to formaldehyde treated virus particles.

probably continuous, sealing over the aperture of the inner component. The inner membrane may well be of different composition as it appears to survive detergent treatment for longer than the outer membrane. This may account for the occasions on which it appears to take a diffuse circular form.

It is still unclear where within the virion the ribonucleic acid is lodged, nor do we know if it is in the form of simple RNA or a more complex ribonucleoprotein. One possibility is that the nucleic acid is contained within the internal 'flask', but alternatively it may lie between this internal component and the outer membrane. It may be suggested that the diffuse, possibly striated, inner circles occasionally observed (Fig. 3) are ribonucleoprotein

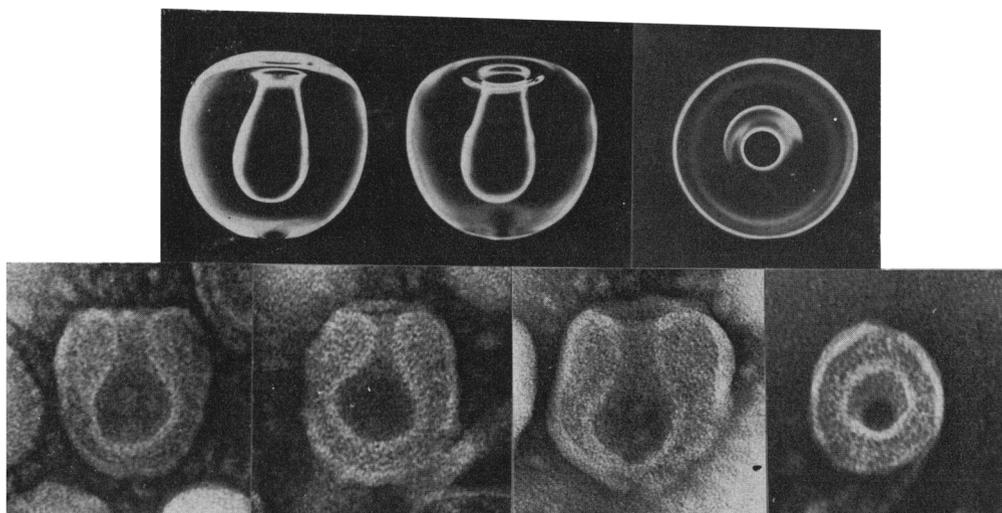


Fig. 7. Three different, transilluminated, orientations of the glass model, are compared to four individual virus particles printed at  $\times 330000$ . Although both refraction and reflection will occur with the glass model the overall correlation is good.

strands and not a membrane. However, these structures are only observed when the membrane is not itself apparent, and moreover have never been observed as separate entities outside the virion. In the gradient fraction of density 1.29 liberated by detergent, only structures resembling the inner membrane were seen. As this density is more typical of a ribonucleoprotein structure, it is possible that the virus ribonucleoprotein is closely associated with the inner membrane and remains firmly integrated with it even after detergent treatment. The comparatively high RNA:protein ratio in that fraction supports this suggestion. It has not yet been possible to isolate sufficient of this material to enable more comprehensive biochemical characterization. Further experiments to this end are in progress.

These results are compatible with the previous reports of an inner shell in coronaviruses, although it seems that in infectious bronchitis virus the shell is not a complete sphere but rather a flask-like structure attached by its 'neck' to the virus envelope. It will be of great interest to see if similar structures are found in other types of coronavirus, or if IBV is unique.

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