

## PRELIMINARY STUDIES ON THE ISOLATION OF CORONAVIRUS 229E NUCLEOCAPSIDS

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### 1. Introduction

Coronaviruses are a large group of enveloped, lipid containing, RNA viruses which are characterised by their distinctive projection morphology [1]. There have been extensive reports describing the morphology of the virion both in thin sections of infected cells and by the negative staining technique [2]. Although several members of this group have been characterized biochemically [3–7] little is known about the morphology of the internal component. Apostolov and Flewett [8] observed 7–8 nm threads in thin sections of pelleted preparations of avian infectious bronchitis virus and Kennedy and Johnson-Lussenburg [9] isolated the internal component of the human respiratory coronavirus 229E and described its morphology from negatively stained preparations. However, Macnaughton et al. [10] recently described the morphology following negative staining of ribonucleoprotein (RNP)-like structures in their preparations of 229E virus which were clearly dissimilar, both in diameter and appearance to those previously reported [9]. Nucleocapsid-like structures, 9 nm in diameter, have been observed in thin sections of organ cultures infected with the human enteric coronavirus [11]. However, before attempting further characterisation of this nucleocapsid-like structure and in view of the conflicting reports of the internal component of 229E virus, we have further examined the internal component of the human coronavirus 229E. This paper presents new evidence on the morphology of its nucleocapsid.

### 2. Material and Methods

#### 2.1. Virus

A laboratory-adapted strain of the respiratory coronavirus 229E was kindly supplied by Dr. P.G. Higgins.

#### 2.2. Cell cultures

A continuous line of human lung cells (MRC-C) were used at passage levels 22–28 in these investigations. These cells were obtained from Dr. S. Reed.

#### 2.3. Preparation of labelled virus

Confluent monolayers of MRC-C cells were infected with 229E virus and simultaneously labelled with 100  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]uridine (spec. act. 5.0 Ci/mmol). (The Radiochemical Centre, Amersham). The cultures were incubated at 35°C and virus was harvested at 36 h post infection when the cytopathic effect was 90–100% complete.

#### 2.4. Concentration of virus

Cultures were frozen and thawed twice prior to clarification at 1500 g for 20 min. Ammonium sulphate was added to the supernatant until 60% saturation was achieved and the preparation was then left at 4°C for 1 h [12]. The precipitated virus was centrifuged at 5000 g for 10 min, and the resultant

deposit redissolved in SET buffer pH 7.5 (0.1 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA).

### 2.5. Purification of virus

The concentrated virus preparation was centrifuged through a discontinuous 10–50% glycerol/potassium tartrate gradient [13] for 90 min at 4°C at 130 000 *g* in an MSE superspeed “75” ultracentrifuge. 1-ml fractions were collected by tube bottom puncture using an LKB “Redirac” fraction collector. Samples of each fraction were trichloroacetic acid (TCA)-precipitated on glass fibre pads (GF/F Whatman) and counted using a toluene based scintillation cocktail in a Unilux I liquid scintillation counter. The resultant peak fractions were isopycally banded in 20–70% sucrose in SET buffer pH 7.5 at 130 000 *g* for 16 h at 4°C. 1-ml fractions were collected and radioactivity was determined as above. Densities were measured using a Bellingham and Stanley refractometer. The fraction containing purified virus (density in sucrose of 1.18 g/cm<sup>3</sup>) was divided in two and mixed with equal volumes of 1% Nonidet-P40 (NP-40) and SET buffer. These mixtures were held at room temperature for 15 min before isopycnic centrifugation in 20–70% sucrose as before.

An egg-adapted strain of Sendai virus, provided by Mr. S. Dunn, was isopycally banded and the 1.27 g/cm<sup>3</sup> fraction containing spontaneously released nucleocapsids, was used for comparative electron microscopy.

### 2.6. Electron microscopy

Formvar-coated grids were prepared either directly from diluted fractions or from fractions dialysed against SET buffer overnight at 4°C. Grids were stained with 1.5% phosphotungstic acid pH 6.5 or 1% uranyl acetate and examined in an AEI 801 electron microscope at an instrument magnification of X63 000.

## 3. Results

The distribution of [<sup>3</sup>H]uridine label in a glycerol tartrate gradient following rate zonal centrifugation is shown in Fig. 1. Electron microscopic examination of

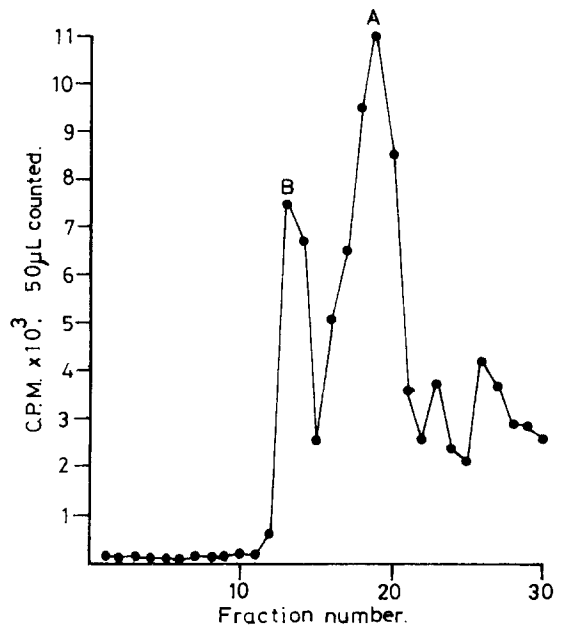


Fig. 1. Rate zonal centrifugation of 229E virus through a 10–50% w/w glycerol/tartrate gradient for 90 min at 130 000 *g*.

the upper peak (A) showed large numbers of typical intact coronavirus particles (Fig. 2). Although significant precipitable radioactive counts were present at a lower peak (B, Fig. 1), no virus particles were ob-

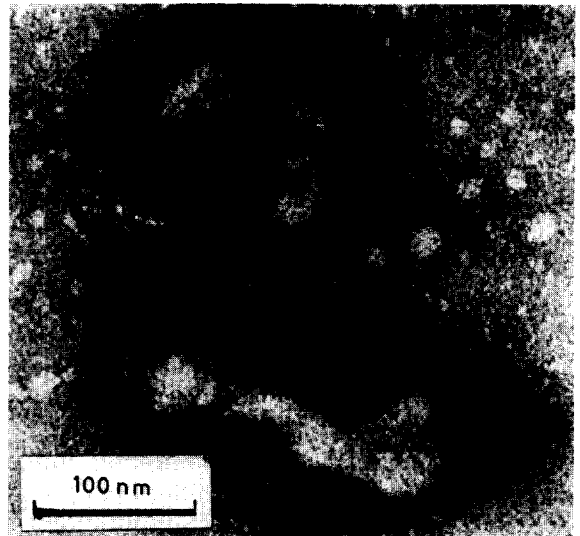


Fig. 2. Phosphotungstic acid-stained virus from peak A (Fig. 1) showing typical intact coronavirus particles.

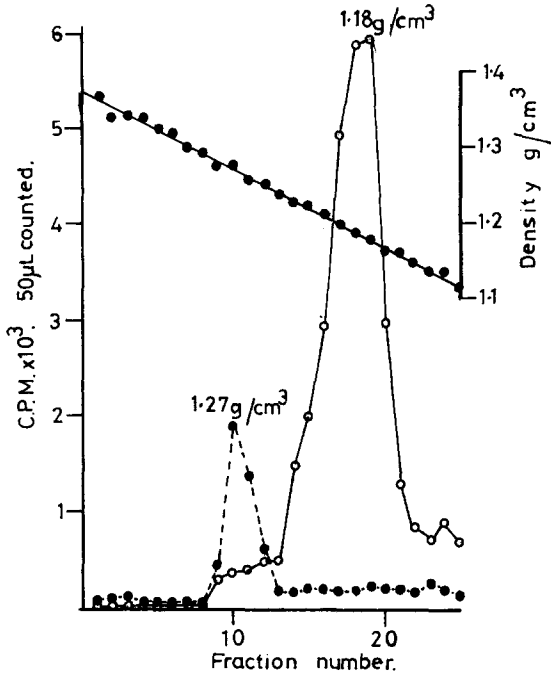


Fig. 3. Equilibrium centrifugation through 20–70% w/w sucrose of material from peak A (○—○) and peak B (●- - -●) and distribution of density (●—●).

served by electron microscopy. This strongly suggested the release of a denser component from disrupted virions or cells. In view of this possibility both peaks were re-banded isopycnicly in sucrose.

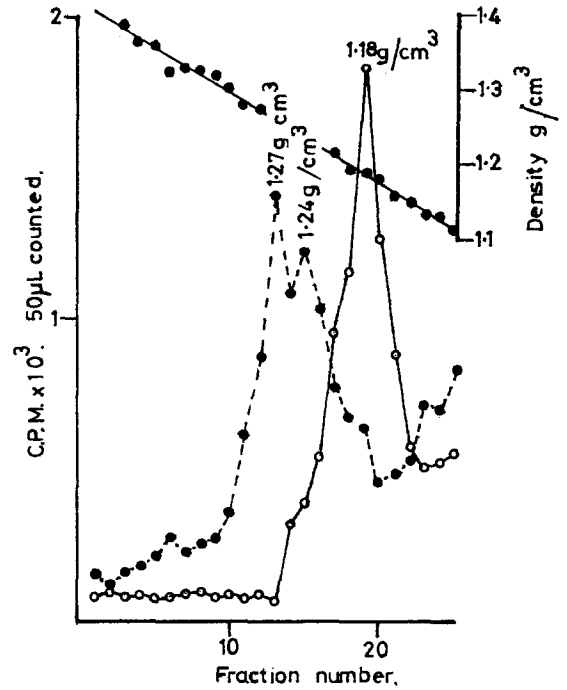


Fig. 4. Equilibrium centrifugation of NP-40 treated 229E virus (●- - -●) and control virus treated with SET buffer (○—○) through 20–70% w/w sucrose. Distribution of density (●—●).

Equilibrium centrifugation of peak (A) (Fig. 1) resulted in a major peak of radioactivity at a density of 1.18 g/cm<sup>3</sup> (Fig. 3). Subsequent examination of

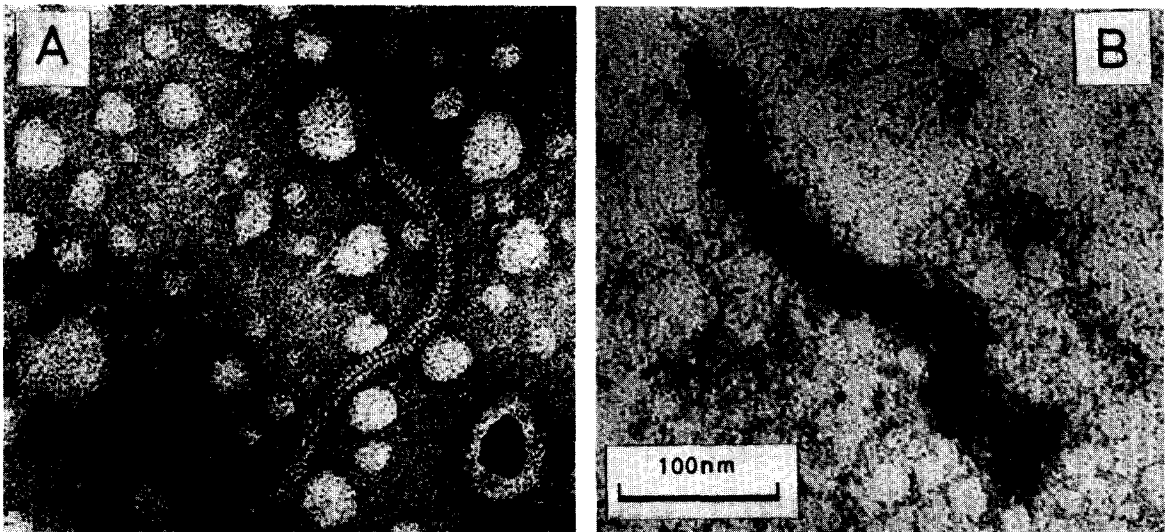


Fig. 5. Uranyl acetate-stained 229E virus nucleocapsid (a) and paramyxovirus nucleocapsid (b) from 1.27 g/cm<sup>3</sup> fractions.

this material by electron microscopy showed typical coronavirus particles similar to those shown in Fig. 2. Simultaneous equilibrium centrifugation of peak (B) resulted in a major peak of radioactivity at a density of  $1.27 \text{ g/cm}^3$  (Fig. 3).

NP-40 treatment of whole purified virus resulted after centrifugation in two peaks of radioactivity with densities of  $1.27$  and  $1.25 \text{ g/cm}^3$  (Fig. 4). Control virus mixed with SET buffer banded as before at a density of  $1.18 \text{ g/cm}^3$ .

Electron microscopic examination of the dialysed  $1.27 \text{ g/cm}^3$  peaks from both the NP-40 treated virus and the dense component resulting from equilibrium centrifugation of the rate zonal peak (B, Fig. 1), which had not been treated with NP-40, revealed linear structures which had a clear periodicity and 3–4 nm central canal (Fig. 5a). The diameter of these structures ranged from 9–11 nm and 11–13 nm following staining with phosphotungstic acid and uranyl acetate respectively. Similar structures were seen in the dialysed  $1.24 \text{ g/cm}^3$  fraction of the NP-40 treated virus in association with densely stained filaments (Fig. 6). The appearance of paramyxovirus nucleocapsids following uranyl acetate staining is shown in Fig. 5b. The diameter of the paramyxovirus nucleocapsids ranged from 18–20 nm.

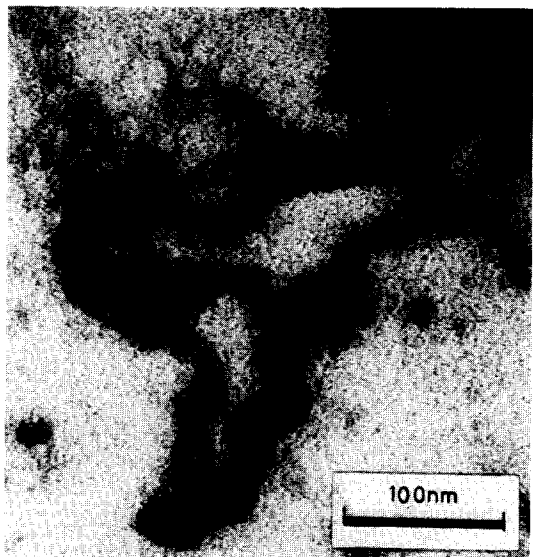


Fig. 6. Electron-dense filamentous structures present in the  $1.24 \text{ g/cm}^3$  fraction in association with 229E virus nucleocapsid. Uranyl acetate-stained.

#### 4. Discussion

Coronavirus 229E was found to have a density in sucrose of  $1.18 \text{ g/cm}^3$  which is compatible with published reports [1]. The structures released by detergent treatment of intact purified virus superficially resembled paramyxovirus nucleocapsids in their overall morphology. However, these nucleocapsid-like structures were significantly smaller in diameter and the appearance of the subunit structure differed from the classical "herring bone" appearance of paramyxovirus nucleocapsids Fig. 5b [14]. The density of  $1.27 \text{ g/cm}^3$  in sucrose for these nucleocapsid-like structures is within the range of reported densities for the nucleocapsids of other large RNA viruses [15].

The purified nucleocapsid-like structures observed here are morphologically different from those previously reported by Kennedy and Johnson-Lussenburg [9] who purified and detergent-treated 229E virus in a similar manner. The differences between the morphology of the previously described structures and those reported here could be explained by the electron microscopical preparative procedures used. The finding of the nucleocapsid-like structures in close association with densely staining filaments could account for the observation of these nucleocapsid-like structures at the lighter density of  $1.24 \text{ g/cm}^3$ . The association of protein with the RNP of other coronaviruses has been reported [16,17].

In order to confirm that the structures we have seen are nucleocapsids it is necessary to determine their protein composition and sensitivity to RNAase. Studies in progress suggest that the nucleocapsid-containing fractions have a major protein with a molecular weight of approx. 45 000 daltons. A protein of approximately this molecular weight has tentatively been assigned to the nucleocapsid structures of coronaviruses [4,7]. Work is continuing to further characterize the nucleocapsid and its polypeptides.

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