

Heterogeneity of Infectious Bronchitis Virus Grown in Eggs

By

M. S. COLLINS¹, D. J. ALEXANDER¹, and J. W. HARKNESS

Poultry¹ and Virology Departments, Central Veterinary Laboratory,
New Haw, Weybridge, Surrey, Great Britain

With 8 Figures

Accepted September 3, 1975

Summary

Egg-grown infectious bronchitis virus, strain Beaudette, was concentrated and centrifuged on sucrose density gradients to separate the virus into five peaks with densities of 1.144, 1.160, 1.172, 1.191 and 1.218 g/cm³. All peaks retained infectivity, complement fixation activity and were labelled with ³H-uridine. Morphologically the densest peak consisted of very large virus particles and amorphous material, the other peaks consisted of mainly intact particles although small differences in size and pleomorphism were seen.

Polyacrylamide gel electrophoresis of material from the density gradient peaks revealed four major polypeptides and at least 10 minor polypeptides. The proportions of the polypeptides were approximately similar for all peaks with the exception of the densest peak in which the major polypeptides were greatly reduced. The four major polypeptides had approximate molecular weights of 1. 52,000, 2. 45,000, 3. 34,000, 4. 32,000. The major polypeptides 1 and 4 were shown to be glycosylated as were two of the minor polypeptides.

Introduction

Avian infectious bronchitis virus (IBV) has been classified as a coronavirus (ALMEIDA *et al.*, 1968). Morphologically it exists as pleomorphic virus particles 80—120 nm in diameter which usually possess projections 20 nm in length and 9—11 nm in width at the outer end (MCINTOSH *et al.*, 1967; BERRY and ALMEIDA, 1968). However, the presence or absence of the projections and their exact morphology has varied with different reports (CUNNINGHAM, 1970). Recently HARKNESS and BRACEWELL (1974) have shown variations in the possession of a corona amongst 12 strains, and have related these to immunological variations. APOSTOLOV *et al.* (1970) examined IBV particles in ultra thin sections which revealed an envelope, similar in appearance to the "unit cell membrane", enclosing an internal thread-like structure 7—8 nm in diameter.

Studies on the morphogenesis of IBV in chick embryo fibroblasts (NAZERIAN and CUNNINGHAM, 1968), chorioallantoic membrane cells (BECKER *et al.*, 1967) or VERO cells (CUNNINGHAM *et al.*, 1972) have produced similar results to those obtained with the Linder strain coronavirus in human embryo lung cells (OSHIRO *et al.*, 1971). This virus was shown to bud into the cisternae or vesicles of the endoplasmic reticulum but occasionally cytoplasmic inclusions of tubular forms were seen which suggest an alternative mode of virus reproduction. BECKER *et al.* (1967) report the complete IBV particle seen in morphogenesis studies as consisting of a double outer shell and a denser inner shell surrounding a core of amorphous material.

There have been few studies on the virion structure of IBV or coronaviruses in general. HEIRHOLZER *et al.* (1972) examined the structural polypeptides of the human coronavirus OC43 and detected a minimum of six polypeptides with a molecular weight range of 15,000—191,000 of which 4 were glycopolypeptides.

Estimations of the buoyant density of IBV have varied considerably: In isopycnic caesium chloride density gradients the density of the Beaudette strain grown in eggs has been reported as 1.23 g/cm³ (TEVETHIA and CUNNINGHAM, 1968), but in sucrose density gradients it was found to be 1.19 (CUNNINGHAM, 1970). MENGELING and CORIA (1972) demonstrated a peak of activity at either 1.198 or 1.205 for IBV on CsCl density gradients but reported a distribution between 1.189—1.220 particularly if the virus had been concentrated by dialysis against polyethylene glycol. TANNOCK (1973) reported a wide distribution of virus in sucrose density gradients with a peak of activity at 1.176. Better resolution was obtained on potassium tartrate density gradients with a peak at 1.16, but tartrate caused high losses of infectivity and disruption of virus particles.

In the present study we have examined the morphological and structural properties of virus particles which differed in buoyant density on sucrose density gradients.

Materials and Methods

Virus

The Beaudette strain of avian infectious bronchitis virus (IBV) was grown in 9 or 10-day-old fertile chicken eggs by inoculating each egg with 100 ELD₅₀ (50 per cent egg lethal doses of virus) by the allantoic route. Infected amnio-allantoic fluids were harvested 40 hours after infection. Identical results were obtained with virus that had been cloned by three passages to limiting dilutions in eggs as well as uncloned virus.

Virus Concentration and Density Gradient Analysis

Virus from harvested fluids was concentrated by centrifugation at 30,000 × *g* for 40 minutes and resuspension in 0.1 M Tris-HCl buffer pH 7.2.

Initial density gradient analysis was by centrifugation at 80,000 × *g* for 2 hours through either a 20 ml 20—55 per cent (w/w) continuous sucrose density gradient or a discontinuous gradient consisting of sucrose solutions of densities: 1.134 (4.5 ml), 1.150 (4.5 ml), 1.164 (4.5 ml), 1.180 (4.5 ml) and 1.26 (2 ml). Four bands from the interfaces of the discontinuous gradient were collected separately and again centrifuged at 80,000 × *g* for 2 hours through the appropriate discontinuous gradient prepared from sucrose solutions with the following densities: a) 1.134 (9 ml), 1.150 (9 ml); b) 1.150 (9 ml), 1.164 (9 ml); c) 1.164 (9 ml), 1.180 (9 ml) and d) 1.180 (8 ml), 1.26 (10 ml).

Final purification and separation was on a continuous sucrose density gradient at $80,000 \times g$ for 16 hours. The material from each peak was reconcentrated and resuspended in 0.1 M Tris-HCl buffer pH 7.2 for protein estimations, infectivity and complement fixation testing or in 0.01 M phosphate buffer pH 7.2 for polyacrylamide gel electrophoresis.

Infectivity Assay

Infectivity was estimated by inoculating 0.1 ml volumes of a ten-fold dilution series of virus suspension into the allantoic cavity of 9 or 10-day-old fertile chicken eggs. Seven eggs were used for each dilution. The eggs were candled daily and the end point expressed as ELD₅₀ calculated by the Spearman-Kärber method.

Radioisotope Labelling

Virus labelled with ³H-uridine was obtained by inoculating eggs with 50 µCi of ³H-uridine immediately after infection. ³H (5)-uridine (20–30 Ci/mM) was obtained from the Radiochemical Centre, Amersham, U.K.

Complement Fixation

The complement fixation test was basically that of BRACEWELL (1973) but was modified so that virus and not antiserum was titrated. A standard serum dilution containing two complement fixation units (CFU) was added to each two-fold dilution of antigen. Chicken antisera specific for IBV strain Beaudette was supplied by C.D. Bracewell, Central Veterinary Laboratory, U.K.

Polyacrylamide Gel Electrophoresis

Polypeptides were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) as described (ALEXANDER, 1974).

Protein Estimation

Protein was estimated by the method of LOWRY *et al.* (1951) using bovine serum albumin as a standard.

Results

Sucrose Density Gradient Analysis

Centrifugation of concentrated virus on linear sucrose density gradients produced a broad spread of material detected by measurement of extinction at 254 nm, ranging in density from 1.12–1.22 g/cm³ and consisting of up to three peaks (Fig. 1). To examine the possibility of structural or morphological differences of virus particles with different buoyant densities, a discontinuous sucrose density gradient was used to separate the virus into four distinct peaks (Fig. 2). Fractions containing the four peaks were taken and re-centrifuged on discontinuous gradients to give clean preparations of each peak. The densest peak could be separated into two distinct peaks at this stage (Fig. 3). Although this was a consistent finding, the amount of material involved in the denser of the two peaks (V) was related to the length of time the virus was stored at temperatures other than –60° C. Material from each of the five separated peaks was centrifuged on a continuous sucrose density gradient for 16 hours at $80,000 \times g$. The results obtained are shown in Figure 4 (a–e). The densities recorded for each peak were: I 1.144, II 1.160, III 1.172, IV 1.191, V 1.218. Peak V was always more heterogeneous in density than the other peaks.

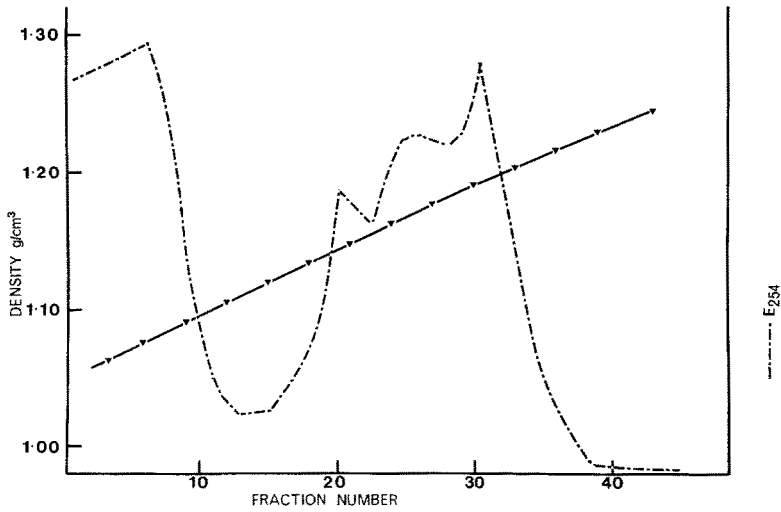


Fig. 1. Linear sucrose density gradient analysis
Concentrated virus was applied to a 20—55 per cent linear sucrose gradient and centrifuged for 2 hours at $80,000 \times g$

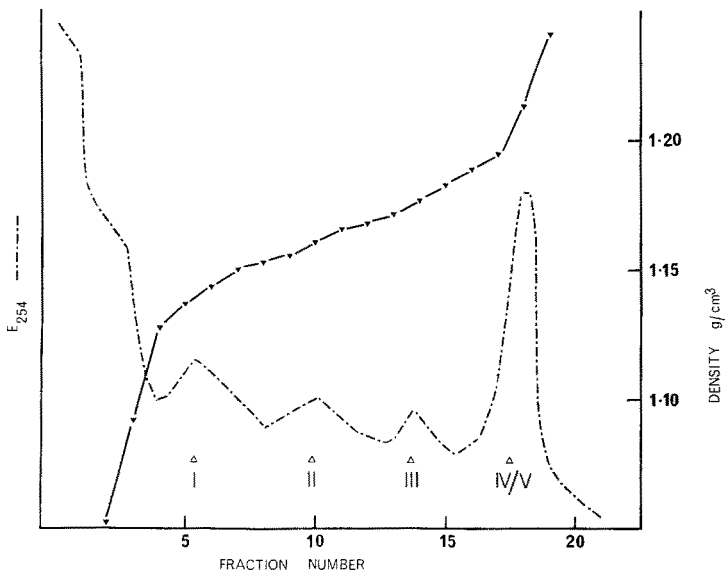


Fig. 2. Discontinuous sucrose density gradient analysis
Concentrated virus was applied to a discontinuous sucrose gradient as described in methods section and centrifuged for 2 hours at $80,000 \times g$

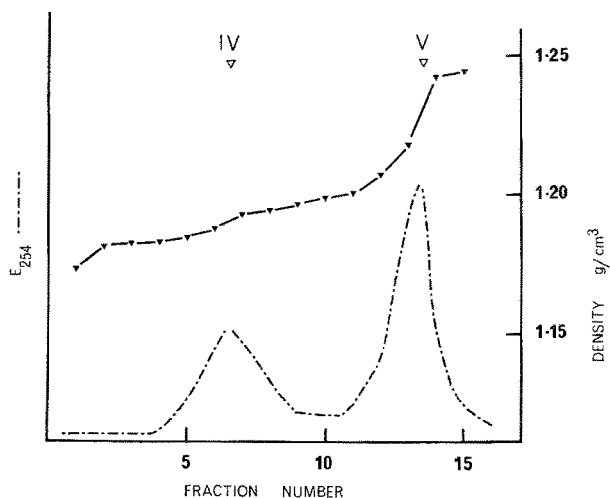


Fig. 3. Density gradient separation of peaks IV and V Peak IV/V from a discontinuous density gradient similar to that shown in Figure 2 was centrifuged for 2 hours at $80,000 \times g$ on a discontinuous sucrose gradient

Properties of the Separated Peaks

Any manipulation of IBV preparations resulted in a large drop in infectivity titre. Although it could be shown that infectivity, complement fixing (CF) activity and ^3H -uridine were associated with all five peaks, titres of infectivity and CF activity were very low. Much higher levels were obtained if the second discontinuous density gradient step in the separation of the peaks was omitted although this meant that peaks IV and V were not separated. Typical results are shown in Table 1.

Some differences in the levels of the activities associated with each peak were detected, in particular I and IV/V showed relatively low levels of CF activity per mg protein but high levels of associated ^3H -uridine. Comparison of CF activity/mg protein in allantoic fluid with peaks II and III from the sucrose density gradients suggest that the virus has been purified more than 400-fold relative to protein.

Table 1. *Properties of peaks from sucrose density gradients*

Peak	CF activity ^a	Infectivity ^b	^3H -uridine activity ^c
Infected allantoic fluid	9	7.26	—
Pelleted virus	969	8.38	—
I	1500	8.56	6882
II	4416	8.60	5716
III	3564	8.62	4500
IV/V	1542	8.19	8005

^a CF units per mg protein

^b log ELD₅₀ per mg protein

^c CPM per mg protein

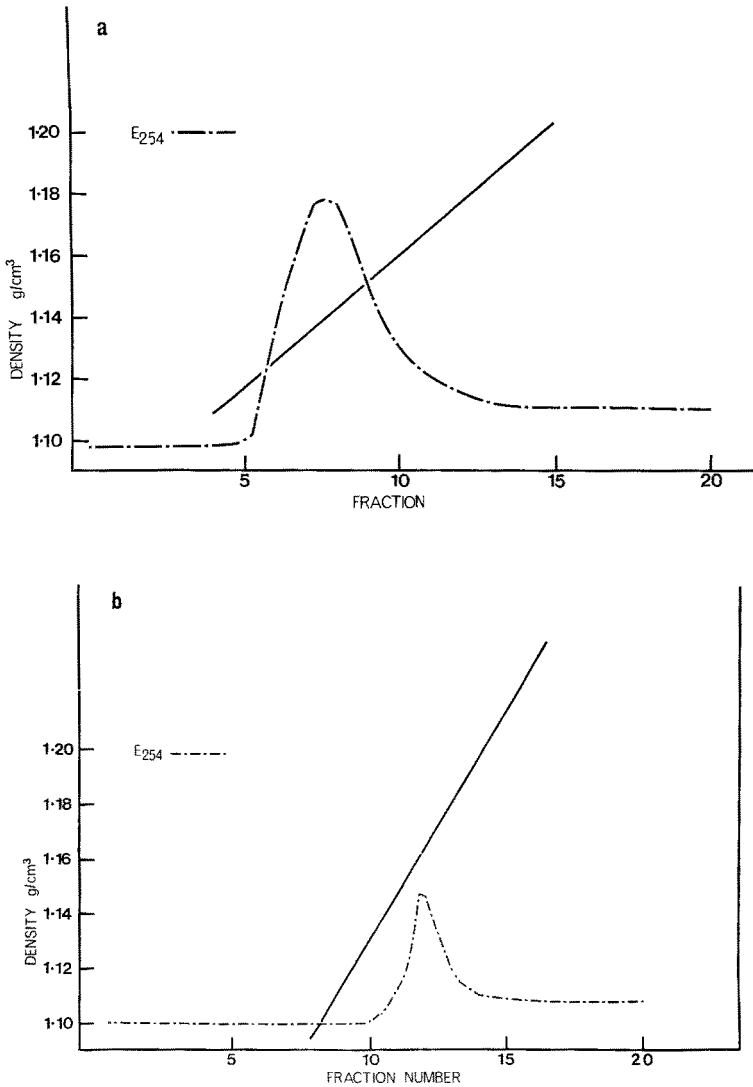


Fig. 4. Isopycnic separation of peaks

Material from each of the five virus-associated peaks was centrifuged for 16 hours at $80,000 \times g$ on continuous sucrose density gradients. a) Peak I, b) Peak II, c) Peak III, d) Peak IV, e) Peak V

Negative-stain electron microscopy revealed several differences in the morphology of the material in each peak (Fig. 5). Peak I consisted of pleomorphic virus particles which were frequently seen as elongated filamentous forms. Filamentous virus particles also formed a small proportion of the virus populations seen in electronmicrographs of peak III but were rarely seen in peak II, which consisted mainly of regular spherical virus particles. Peak IV consisted of particles similar in morphology to those of peak III but with a much larger size range, occasionally

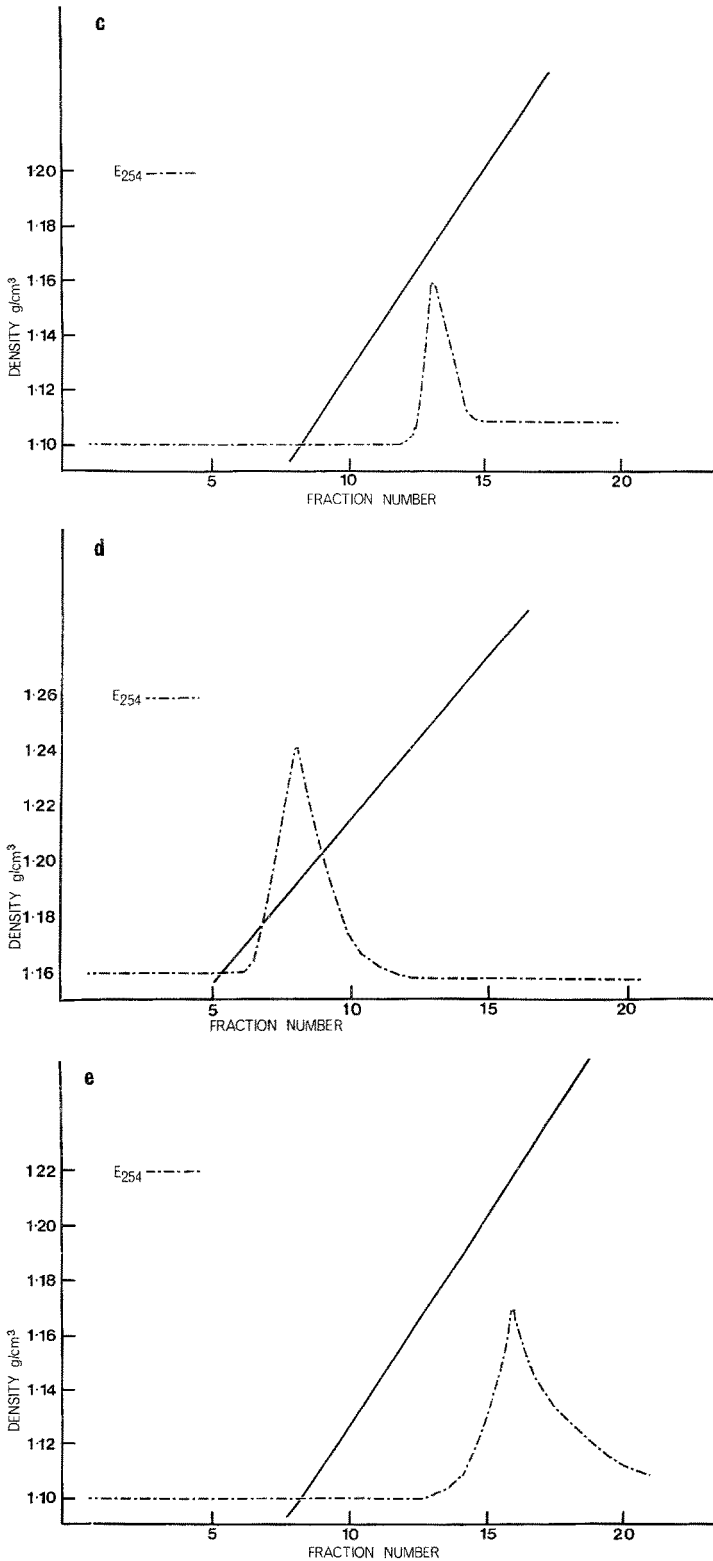


Fig. 4

very large particles were seen in this peak. Peak V consisted mainly of amorphous material although clumps of large particles were also present. The amorphous material was usually in large aggregates but occasionally individual pieces were seen which, although lacking marked structural definition, were distinctive in appearance (Fig. 5e inset). The mean diameters of the virus particles in each peak were: I: 113 ± 23 nm, II: 97 ± 19 nm, III: 112 ± 24 nm, IV: 122 ± 22 nm, V: 182 ± 70 nm. In each peak particles that possessed projections could be seen (Fig. 5a—e). These projections were unlike those usually associated with coronaviruses and rarely formed a complete corona. However, the presence of projections is in contrast with the work of HARKNESS and BRACEWELL (1974) who reported the absence of projections on virions of this strain of IBV.

The possibility existed that the original seed virus may have been a heterogeneous population of genetically different viruses which were merely separated from each other by sucrose gradient centrifugation. This possibility was eliminated

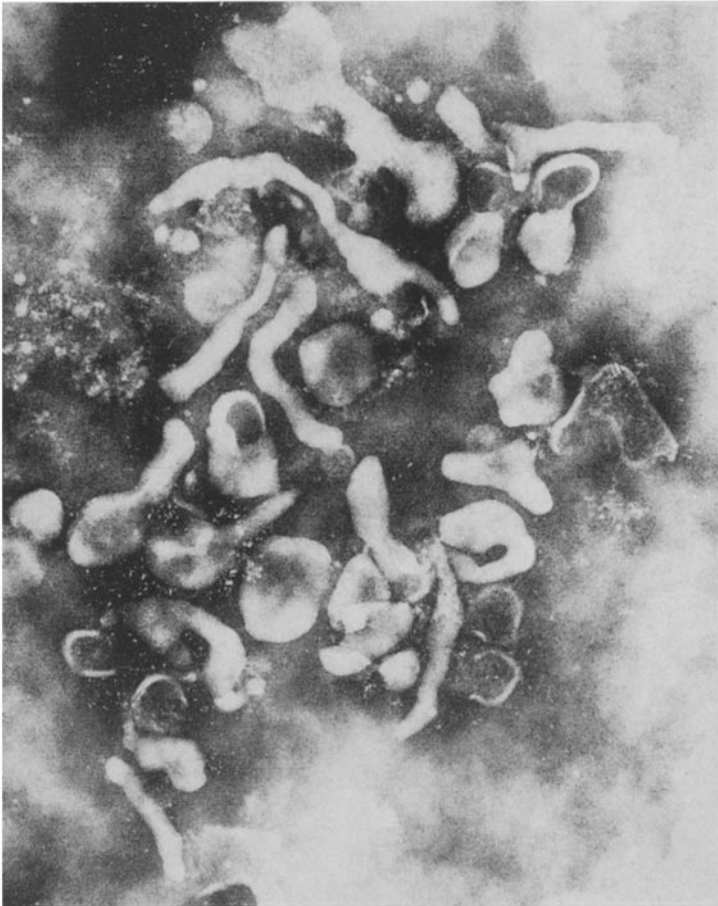


Fig. 5 a

Fig. 5. Electron microscopy of material from sucrose density gradients
a) Peak I, b) Peak II, c) Peak III, d) Peak IV, e) Peak V—inset shows detail of
amorphous material. Magnification $\times 100,000$

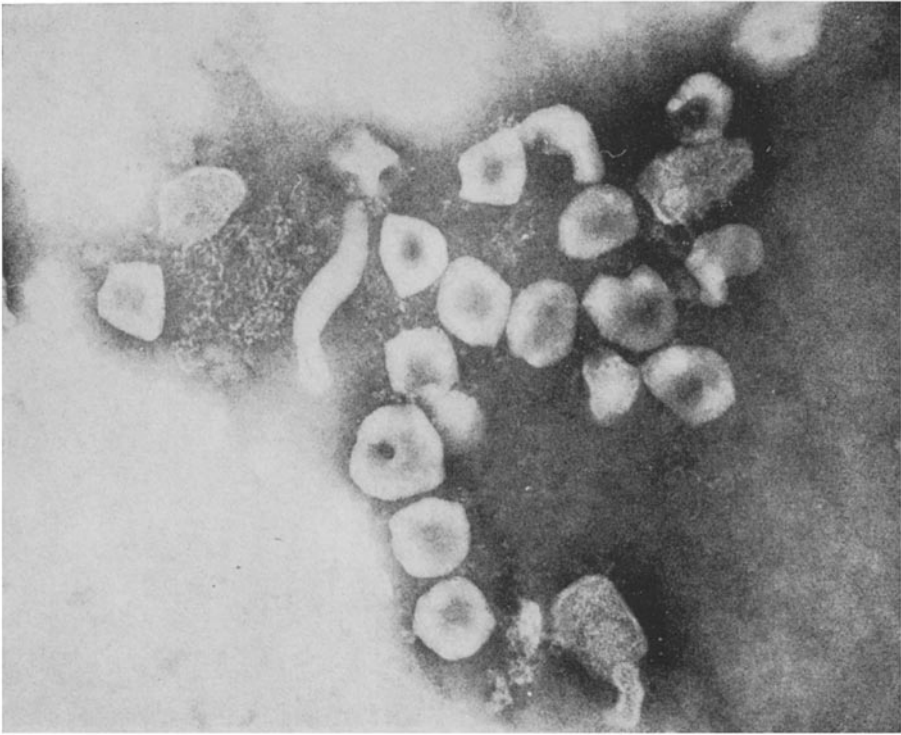


Fig. 5 b

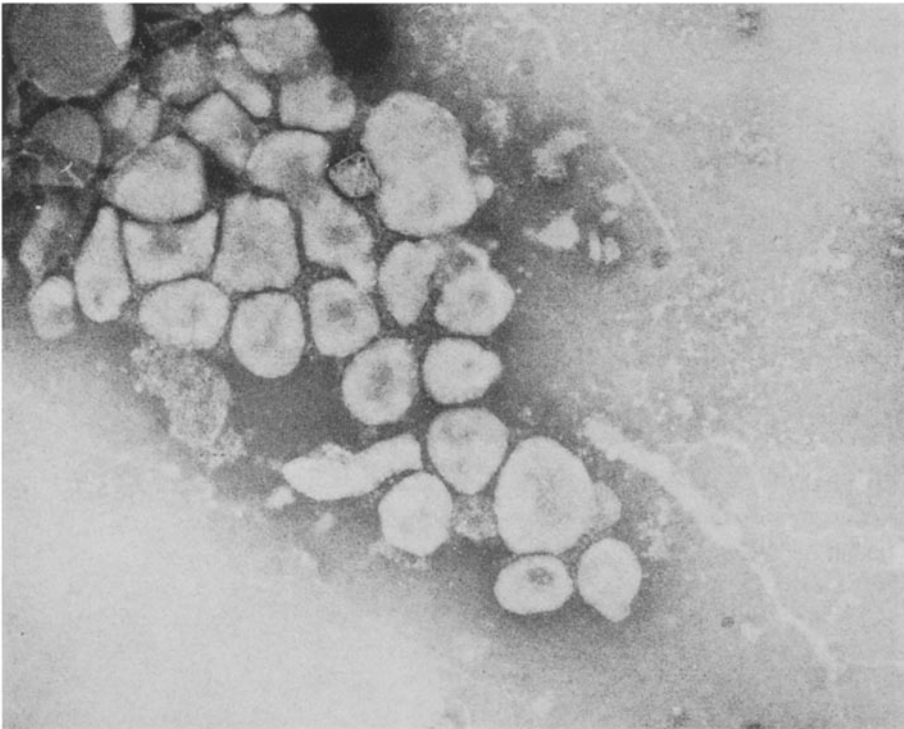


Fig. 5 c

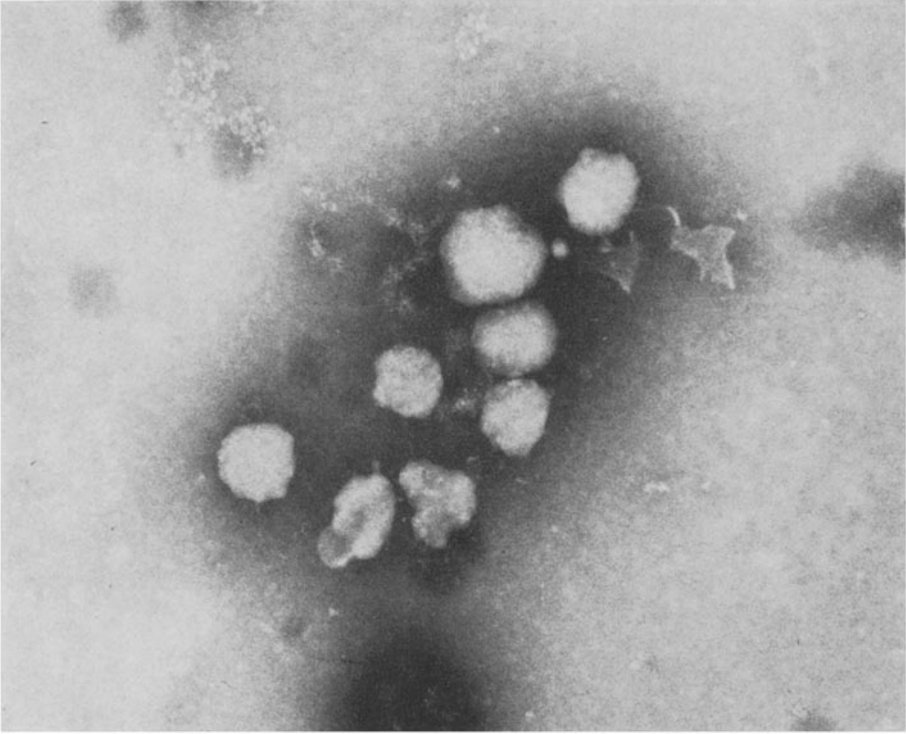


Fig. 5 d

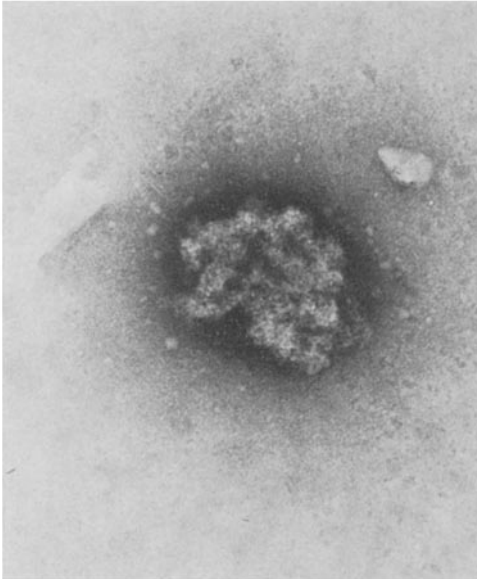


Fig. 5 e (inset)



Fig. 5 e

by titrating the virus from each peak, harvesting the amnio-allantoic fluid from the dead eggs at the highest dilution and passaging this material through eggs. The virus pools produced were concentrated and subjected to sucrose gradient analysis. The virus grown from each of the peaks was also heterogeneous and could be separated into four peaks on discontinuous density gradients.

Polyacrylamide Gel Electrophoresis (PAGE)

Material from each of the five density gradient peaks was disrupted with SDS and dithiothreitol (DTT) and analysed by SDS-PAGE. Staining with amido-black revealed a similar pattern for each of the five peaks (Figs. 6 and 7). In gels of material from peaks I—IV four major polypeptides were evident (1—4) which accounted for more than 70 per cent of the total protein, 40—50 per cent of the total protein was present as polypeptide 1 (Table 2). Polypeptide 3 appeared as a shoulder on polypeptide 4 in gels of material from peaks I-IV but could be seen as a separate polypeptide in gels of peak V where polypeptide 4 was greatly reduced.

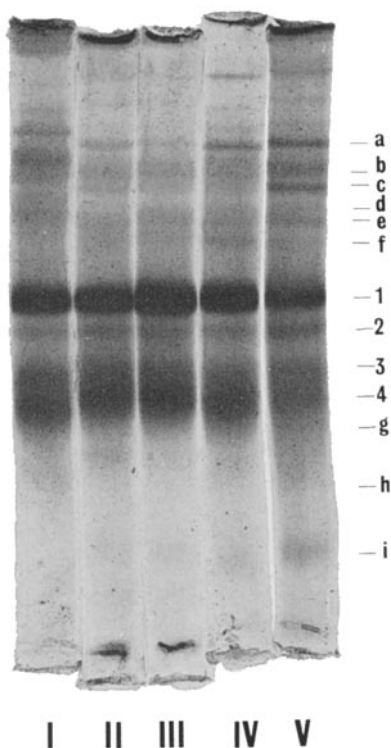


Fig. 6. Separation of the polypeptides from density gradient peaks. Material from each of the sucrose density gradient peaks was disrupted with 1 per cent SDS and 2 per cent DTT and separated by polyacrylamide gel electrophoresis. Gels were stained with amidoblack. The high molecular weight bands seen nearer the origin than band a were not always apparent in stained gels

The approximate molecular weights of the major polypeptides were:

1. 52,000; 2. 45,000; 3. 34,000; 4. 32,000 (Table 2).

Eight or nine minor polypeptides (a—i) were also detectable in gels of all five peaks (Fig. 7). The proportions of the polypeptides were similar for material from all the density gradient peaks with the exception of peak V. In gels of peak V material much higher levels of the minor polypeptides were detected (Fig. 7, Table 2). Occasionally two additional minor bands could be seen in stained gels. These bands had approximate molecular weights of 130,000 and 150,000 but were not usually detected by densitometer scanning.

Polyacrylamide gel electrophoresis of material from the density gradient peaks and staining with Schiff's reagent revealed four bands, A—D (Fig. 8). Band B was present as a minor band which could not always be detected in different preparations of the peaks. The apparent molecular weights of the four bands were: A, 94,000; B, 78,000; C, 52,000; and D, 32,000 (Table 3). Bands C and D corresponded in migration to the major polypeptides 1 and 4 and B corresponded to the minor polypeptide e, this indicates that these three are glycopolypeptides. Band A did not correspond to any amidoblack-stained polypeptide and staining

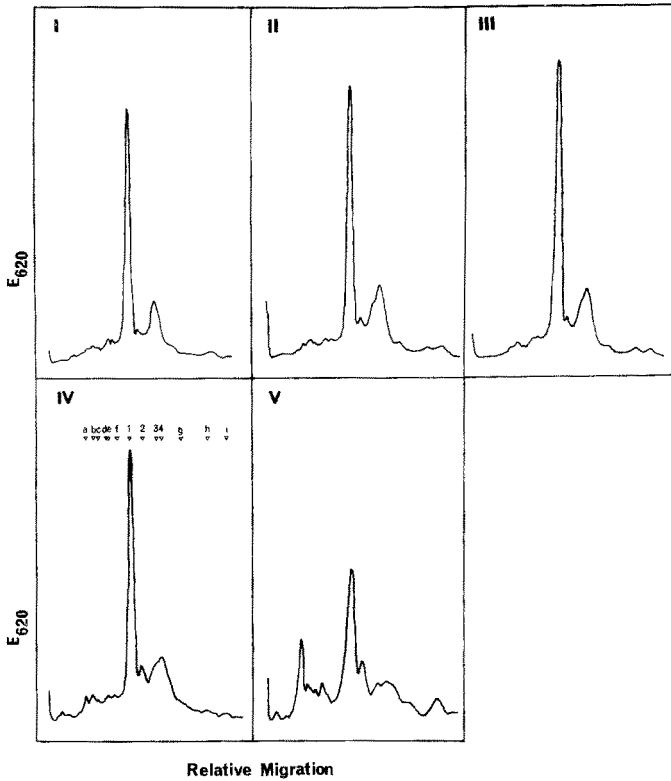


Fig. 7. Polypeptide analysis

Material from each of the sucrose density gradients was treated as described in Figure 6. Gels were stained with amidoblack and scanned at 620 nm. The origin is on the left and the anode on the right

Table 2. Polypeptides detected by PAGE

Poly-peptide	Density gradients peaks									
	I		II		III		IV		V	
	MW ^a	% ^b	MW	%	MW	%	MW	%	MW	%
a	108	}13.3	108	}13.2	108	}10.3	109	}11	116	}22.0
b	101		100		101		101			
c	90		89		89.5		90		92	
d	—		—		—		82		83	
e	76		78		75		76		79	
f	69		70		69		69		69	
1	53	47.5	52	41.0	52.5	49.3	53	46.0	52	25.0
2	45	6.2	46	6.7	44	6.5	45	6.8	44	8.7
3	? ^c	}21.0	?	}26.5	?	}25.5	35	7.8	35.5	6.3
4	33		32.5		31.5		32	20.2	32	12.9
g	25	}12.0	25	}12.6	25	}8.4	24	}8.2	25	}23.7
h	15		16		16		16		18	
i	12		12		12		12		12	

^a Molecular weight $\times 10^{-3}$

^b Per cent total protein

^c Polypeptide 3 masked by polypeptide 4

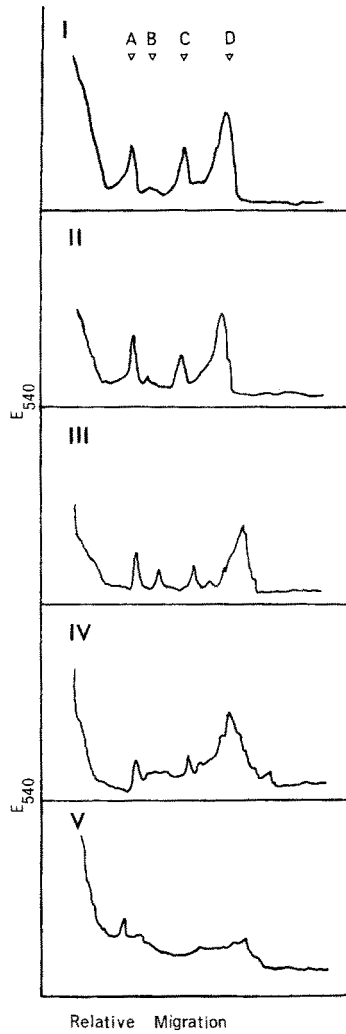


Fig. 8. Glycopolyptide analysis
 Electrophoresis conditions were identical to those for polypeptide analysis (Fig. 7).
 Gels were stained with Schiff's reagent and scanned at 540 nm

Table 3. *The apparent molecular weights of the Schiff—
 positive bands detected by PAGE*

Band	Apparent molecular weight $\times 10^{-3}$ of polypeptides from density gradient peaks				
	I	II	III	IV	V
A	94	93	93	96	96
B	78	78	76	—	—
C	49	52	52	52	—
D	31	31.5	32	31.5	32

with Schiff's reagent followed by amidoblack staining showed that although bands B, C and D were superimposed by bands e, 1, and 4 respectively, band A migrated between polypeptides b and c, as suggested by the molecular weights. Glycopolypeptides which can be stained with Schiff's reagent but not with polypeptide stain have been reported by FAIRBANKS *et al.* (1971) in a study of the polypeptides of the human erythrocyte membrane.

Discussion

Our results show that egg-grown IBV, strain Beaudette, produced a broad density distribution of virus particles on continuous sucrose gradients. Use of discontinuous density gradients resolved the virus material into five distinct peaks, all retaining infectivity and CF activity. HOSAKA *et al.* (1966) obtained similar results with Sendai virus, but were able to show, on a morphological basis, that the variations in density were directly related to the RNA content of the different particles. If the ^3H -uridine activity associated with the density gradient peaks was representative of virus RNA, our results suggest that the differences in density of IBV particles are not related to differences in RNA content. Although the densest peak IV/V had four times the amount of ^3H -uridine cpm per CF unit compared to peaks II and III, peak I, the least dense, had an equally high cpm/CF units ratio. It seems possible, due to the presence of amorphous material and high nucleic acid to protein and CF activity ratios, that peak V, the densest peak, consists of internal virus material from disrupted particles plus very large intact virus particles—these particles could contain more than one genome. In contrast, peaks I—IV consisted of apparently whole infectious virus particles. Additional evidence that the amorphous material may be ribonucleoprotein or similar virus material is the morphological resemblance to the ribonucleoprotein of some oncornaviruses (BOLOGNESI *et al.*, 1973).

The possibility exists that not all contaminating cellular material was removed by the sucrose density gradient centrifugation procedures employed in this study. However, identical PAGE patterns were consistently produced using different preparations of virus; the PAGE patterns of each peak (with the exception of peak V) were very similar; examination of the peaks by electron microscopy revealed relatively homogeneous populations with no obvious cellular material present; PAGE in the absence of SDS and DTT produced no bands for any of the peaks; all of which suggest that if cellular material was present, it was bound to or incorporated into the virus particles. Any assessment of freedom from contaminating cellular material is difficult with a virus as labile as IBV. Populations of coronaviruses have physical to infective particle ratios which may be as high as 10^4 (J. D. ALMEIDA, quoted by BRADBURNE, 1972), for this reason we have used CF units rather than infectivity to give a more realistic comparison of the virus purity. The four hundred-fold purification recorded for peaks II and III compares favourably to the levels of purity obtained using Newcastle disease virus from allantoic fluid (REEVE and ALEXANDER, 1970).

Studies on the morphogenesis of coronaviruses have revealed two possible mechanisms for release: budding from either the membranes of the endoplasmic reticulum or cytoplasmic vesicles, but particles have not been seen to bud from

the plasma membrane (HAMRE *et al.*, 1967; OSHIRO *et al.*, 1971; NAZERIAN and CUNNINGHAM, 1968; BRADFUTE *et al.*, 1970; VETTERLEN and LIEBERMAN, 1970; BECKER *et al.*, 1967; DAVID-FERREIRA and MANAKER, 1965; RUEBNER *et al.*, 1967). However some modification of the cell surface may take place since syncytial formation occurs in cells infected with IBV (AKERS and CUNNINGHAM, 1968). BERRY and ALMEDIA (1968) demonstrated by electron microscopic examination of virus-antibody-complement interactions that the envelopes of IBV particles were derived from the host cell. It is probable that the coronaviruses, like other envelope viruses have a lipid composition resembling that of the host membrane from which they bud (KLENK and CHOPPIN, 1969, RENKONEN *et al.*, 1971) and therefore like Newcastle disease virus (STENBECK and DURAND 1963) would be expected to exhibit host specific differences in buoyant density. If this is the case then two possibilities may exist. Firstly, IBV virions budding from the membranes of different organelles may possess the qualitative and quantitative differences in lipid composition seen in these membranes (ROUSER *et al.*, 1968). Secondly, IBV grown in the embryonated egg may be produced by budding from the endoplasmic reticulum of cells from more than one organ or tissue. Analytical studies of the lipids from the endoplasmic reticulum of different organs have clearly demonstrated marked variations in composition (ROUSER *et al.*, 1968). In this respect it is interesting to note that the density distribution of the coronavirus OC43 which had been adapted to a single organ, the suckling mouse brain, extended only over the range 1.18—1.20 g/cm³ on continuous sucrose gradients (KAYE *et al.*, 1970).

Our results suggest that four major and ten minor polypeptides are associated with IBV virions. This compares favourably with the 16 polypeptides detected in preparations of this virus by BINGHAM (personal communication). If both the major and minor polypeptides represent the structural polypeptides of the virus, then IBV is unlike coronavirus OC43 which has been reported to contain only 6 or 7 polypeptides (HIERHOLZER *et al.*, 1972). Although the apparent IBV structural proteins are within the approximate coding capacity of the large genome suggested for IBV by WATKINS *et al.* (1975), it seems more probable that, unlike the paramyxoviruses, IBV virions incorporate host polypeptides as well as lipids [the work of BERRY and ALMEDIA (1968) suggests that IBV has a host-derived lipoprotein membrane], or host material becomes closely bound to the virus particles. Either case may account for some, or all, of the minor polypeptides seen in this study.

Acknowledgments

We thank Mr. N. J. Chettle for his excellent assistance.

References

1. ALEXANDER, D. J.: Comparison of the structural polypeptides of four avian paramyxoviruses. *Arch. ges. Virusforsch.* **46**, 291—301 (1974).
2. ALMEIDA, J. D., BERRY, D. M., CUNNINGHAM, C. H., HAMRE, D., HOFSTAD, M. S., MALLUCCI, L., MCINTOSH, K., TYRRELL, D. A. J.: Coronaviruses. *Nature (Lond.)* **220**, 650 (1968).
3. APOSTOLOV, K., FLEWETT, T. H., KENDAL, A. P.: In: BARRY, R. D., MAHY, B. W. J. (eds.), *The biology of large RNA viruses*, 3—26. London-New York: Academic Press 1970.

4. AKERS, T. G., CUNNINGHAM, C. H.: Replication and Cytopathogenicity of avian infectious bronchitis virus in chick embryo kidney cells. *Arch. ges. Virusforsch.* **25**, 30—37 (1968).
5. BECKER, W. B., MCINTOSH, K., DEES, J. H., CHANOCK, R. M.: Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 229E). *J. Virol.* **1**, 1019—1027 (1967).
6. BERRY, D. M., ALMEIDA, J. D.: The morphological and biological effects of various antisera on avian infectious bronchitis virus. *J. gen. Virol.* **3**, 97—102 (1968).
7. BOLOGNESI, D. P., LUFTIG, R., SHAPER, J. H.: Localization of RNA tumour virus polypeptides. 1. Isolation of further virus substructures. *Virology* **56**, 549—564 (1973).
8. BRACEWELL, C. D.: A direct complement fixation test for infectious bronchitis virus using heat inactivated chicken sera. *Vet. Res.* **92**, 452—454 (1974).
9. BRADBURN, A. F.: An investigation of the replication of coronaviruses in suspension cultures of L132 cells. *Arch. ges. Virusforsch.* **37**, 297—307 (1972).
10. BRADFUTE, O. E., BOHL, E. H., HARADA, K.: Quoted in: DUNNE, H. (ed.), *Diseases of swine*, p. 161. 3rd ed. Iowa State Univ. Press (1970).
11. CUNNINGHAM, C. H., AVIAN infectious bronchitis. *Adv. vet. Sci. comp. Med.* **14**, 105—148 (1970).
12. CUNNINGHAM, C. H., SPRING, M. P., NAZERIAN, K.: Replication of avian infectious bronchitis virus in African green monkey kidney cell line VERO. *J. gen. Virol.* **16**, 423—427 (1972).
13. DAVID-FERREIRA, J. F., MANAKER, R. A.: An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. *J. Cell Biol.* **24**, 57—78 (1965).
14. FAIRBANKS, G., STECK, T. L., WALLACH, D. F. H.: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606—2617 (1971).
15. HAMRE, D., KINDIG, D. A., MANN, J.: Growth and intra-cellular development of a new respiratory virus. *J. Virol.* **1**, 810—816 (1967).
16. HARKNESS, J. W., BRACEWELL, C. D.: Morphological variation among avian infectious bronchitis virus strains. *Res. vet. Sci.* **16**, 128—131 (1974).
17. HIERHOLZER, J. C., PALMER, E. L., WHITFIELD, S. G., KAYE, H. S., DOWDLE, W. R.: Protein composition of coronavirus O C43. *Virology* **48**, 516—527 (1972).
18. HOSAKA, Y., KITANO, H., IKEGUCHI, S.: Studies on the pleomorphism of HVJ virions. *Virology* **29**, 205—221 (1966).
19. KAYE, H. S., HIERHOLZER, J. C., DOWDLE, W. R.: Purification and further characterisation of an "IBV-like" virus (coronavirus). *Proc. Soc. exp. Biol. Med.* **135**, 457—463 (1970).
20. KLENK, H.-D., CHOPPIN, P. W.: Lipids of plasma membranes of monkey and hamster kidney cells and of parainfluenza virus grown in these cells. *Virology* **38**, 255—268 (1969).
21. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., RANDALL, R. J.: Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* **193**, 265—275 (1951).
22. MCINTOSH, K., DEES, J. H., BECKER, W. B., KAPIKIAN, A. Z., CHANOCK, R. M.: Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc. nat. Acad. Sci. U.S.A.* **57**, 933—940 (1967).
23. MENGELING, W. L., CORIA, M. F.: Buoyant density of hemagglutinating encephalomyelitis virus of swine; comparison with avian infectious bronchitis virus. *Amer. J. vet. Res.* **33**, 1359—1363 (1972).
24. NAZERIAN, K., CUNNINGHAM, C. H.: Morphogenesis of avian infectious bronchitis in chicken embryo fibroblasts. *J. gen. Virol.* **3**, 469—470 (1968).
25. OSHIRO, L. S., SCHIEBLE, J. H., LENNETTE, E. H.: Electron microscopic studies of coronavirus. *J. gen. Virol.* **12**, 161—168 (1971).
26. REEVE, P., ALEXANDER, D. J.: Isopycnic separation of Newcastle disease virus on sucrose gradients using zonal ultracentrifuge rotors. *Microbios* **2**, 53—58 (1970).

27. RÄNKÖNEN, O., KÄÄRÄINEN, L., SIMONS, K., GAMMBERG, C. C.: The lipid class comparison of Semliki forest virus and of plasma membranes of the host cells. *Virology* **46**, 318—326 (1971).
28. ROUSER, G., NELSON, G. J., FLEISCHER, S.: Lipid composition of animal cell membranes organelles. In: CHAPMAN, D. (ed.), *Biological membranes—physical fact and function*, 5—69. London-New York: Academic Press 1968.
29. RUEBNER, B. H., HIRANO, T., SLUSSER, R. J.: Electron microscopy of the hepatocellular and kupffer cell lesions of mouse hepatitis with particular reference to the effect of cortisone. *Amer. J. Pathol.* **51**, 163—190 (1957).
30. STENBECK, W. A., DURAND, D. B.: Host influence on the density of Newcastle disease virus. *Virology* **20**, 545—551 (1963).
31. TANNOCK, G. A.: The nucleic acid of infectious bronchitis virus. *Arch. ges. Virusforsch.* **43**, 259—271 (1973).
32. TEVETHIA, S. S., CUNNINGHAM, C. H.: Antigenic characterisation of infectious bronchitis virus. *J. Immunol.* **100**, 793—798 (1968).
33. VETTERLEIN, W., LIEBERMANN, H.: The morphological development of the virus of transmissible gastroenteritis as compared to other viruses. *Arch. exp. Vet.-Med.* **24**, 549—558 (1970).
34. WATKINS, H., REEVE, P., ALEXANDER, D. J.: The ribonucleic acid of infectious bronchitis virus. *Arch. Virol.* **47**, 279—286 (1975).

Authors' address: Dr. D. J. ALEXANDER, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, Great Britain.

Received April 24, 1975