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# The Ribonucleic Acid of Infectious Bronchitis Virus

By

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With 5 Figures

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## Summary

Analysis of the nucleic acid of infectious bronchitis virus by SDS polyacrylamide gel electrophoresis revealed an RNA of molecular weight $9.0 \times 10^6$ Daltons. The RNA was shown to have a sedimentation coefficient of 50.

## 1. Introduction

Infectious bronchitis virus (IBV) is a member of the group of RNA-containing enveloped viruses termed Coronaviruses. In man, viruses of this group may cause common-cold-like diseases and in pigs two fatal diseases are associated with coronavirus infections. IBV infections may cause considerable morbidity in adult chickens, affecting egg production and growth rates, and cause high mortality in young chicks (9). In spite of the importance and wide distribution of these pathogens little is known of the virus structure.

Morphological studies (3, 4, 5) have shown that the virions of IBV are pleomorphic particles 80—120 nm in diameter, with an internal component 7—8 nm in cross-section, bounded by an envelope membrane which in most strains has a characteristic corona of spikes.

Studies on the structural polypeptides of a human coronavirus, OC43, revealed 6 or 7 polypeptides with molecular weights of 191,000-15,000 (11), but studies with IBV have shown a more complex picture (COLLINS, ALEXANDER and HARKNESS, in preparation).

A recent study of the RNA of IBV (15) has suggested that the RNA consists of a discontinuous single strand with extreme heterogeneity in the sizes of the RNA fragments as revealed by centrifugation on sucrose gradients, or polyacrylamide gel electrophoresis.

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In contrast ,we describe the extraction of a single, large molecular weight species of RNA from purified IBV virions, using a method described by KINGSBURY (12) for isolation of Newcastle disease virus RNA.

## 2. Materials and Methods

### *2.1. Radioisotopes and Other Materials*

The following radioisotopes were purchased from the Radiochemical Centre, Amersham, U.K.: Carrier free $^{32}P$ orthophosphate (10 mCi/ml), $^{3}H$-L-Leucine (46 Ci/mM), 5-$^{3}H$ Uridine (20—30 Ci/mM).

Uridine 2,3 monophosphate (UMP) was obtained from Sigma Ltd., ribonuclease was from B.D.H. Ltd., Poole, England.

### *2.2. Virus Preparation and Purification*

Ten-day-old fertile chicken eggs were inoculated with 100—1000 $ELD_{50}$ IBV (Beaudette strain). Approximately 2 hours later, 50—100 μCi $^{32}P$ and 20—50 μCi $^{3}H$-L-Leucine were inoculated into each embryo. In preliminary experiments embryos were inoculated with 20—50 μCi $^{3}H$-Uridine after virus inoculation. After 30 hours incubation at 37° C, infected eggs were chilled and the allantoic fluid harvested. After low speed centrifugation to remove cell debris, the virus was concentrated by centrifugation at 40,000 g for 40 minutes. The pellet was resuspended in 0.01 M Tris-EDTA buffer pH 7.4 (TE buffer). Purification was by centrifugation through 37 per cent (w/w) sucrose at 40,000 g for one hour and the pellet was resuspended in TE buffer. Further purification was done by isopycnic separation on linear 0—60 per cent (w/w) sucrose gradients, by centrifugation for 2 hours at 25,000 r.p.m., or on discontinuous gradients consisting of 60, 55 and 38 per cent sucrose. A $3 \times 20$ ml rotor was used throughout. The virus band was collected, diluted with TE buffer and pelleted at 40,000 g for 40 minutes. The final pellet was resuspended in TE buffer and used immediately or stored at —70° C.

### *2.3. Infectivity and Complement Fixing Activity*

Infectivity was estimated as 50 per cent Egg lethal dose ($ELD_{50}$) in 9 to 10-day old eggs as described (2). Complement fixing activity was measured by the method of BRACEWELL (7).

### *2.4. RNA Markers*

Ribosomal RNA from *E. coli*, and rat liver were extracted by the method of KIRBY (13) and precipitated with 99 per cent ethanol. The RNA was dried with an ether/ethanol mixture, made up to 5 mg/ml in 0.1 M sodium acetate buffer pH 5.2 and stored at —20° C. Transfer (5S) RNA was obtained from Miles Products.

### *2.5. SDS Sucrose Gradient Centrifugation*

Samples of radio-isotope-labelled IBV were made 1 per cent with respect to SDS and heated for 2 minutes at 60° C. Samples of virus and *E. coli* ribosomal RNA were layered onto preformed linear 15—30 per cent (w/w) sucrose gradients containing 1 per cent SDS. Rat liver ribosomal RNA and 5S RNA were run simultaneously on identical gradients. Centrifugation was either for one hour at 50,000 r.p.m. in a $3 \times 6.5$ ml titanium rotor or for 16 hours at 22,000 r.p.m. in a $3 \times 20$ ml rotor.

Gradients were removed by upward displacement using an ISCO density gradient remover and 12 or 25 drop fractions were collected. Total radioactivity was estimated by dissolving 10 or 20 μl samples in Oxitol scintillation fluid (1) and counting on an ICN Tracerlab Corumatic 200 Spectrometer. Acid-insoluble radioactivity was measured by precipitating the remainder of the fractions with 5 per cent (w/v) trichloracetic acid (TCA) in the presence of 50 μg carrier RNA. The precipitates were washed with 20 ml ice-cold 5 per cent TCA on glass fibre circles (Whatman Ltd.), dried, and the radioactivity estimated.

### *2.6. Polyacrylamide Gel Electrophoresis (PAGE)*

Polyacrylamide gels 7 mm in diameter and 80 mm in length were cast in glass tubes by polymerising a solution containing: 2.2 per cent (w/v) acrylamide, 0.125 per cent (w/v) N, N-methylenebisacrylamide, 0.03 per cent (v/v) N, N, N', N'-tetramethylethylenediamine, 10 per cent (v/v) glycerol, in 0.125 M Tris-HCl buffer pH 7.0. Samples, or marker RNA, were made 1 per cent with SDS, 20 per cent with glycerol and 0.01 per cent with bromophenol blue and layered onto the surface of the gels. The resevoir buffer was 0.04 m Tris-acetate pH 7.8, containing 0.1 per cent SDS, 10 per cent glycerol and 2 mM UMP. Electrophoresis was at 5—7 mA per gel for approximately 4 hours. After fixing in ice cold 5 per cent TCA, gels were scanned on a Joyce-Loebl UV scanner, sliced, and radioactivity estimated as described (1).

## 3. Results

### *3.1. Purification of IBV*

Purification of virus labelled with $^3$H-Uridine on linear sucrose gradients produced a single peak of radioactivity at an approximate density ($D_{20°}$) of 1.18 g/cm$^3$. Infectivity and complement fixing activity were related to this peak, confirming that IBV is an RNA-containing virus (Figs. 1 and 2).

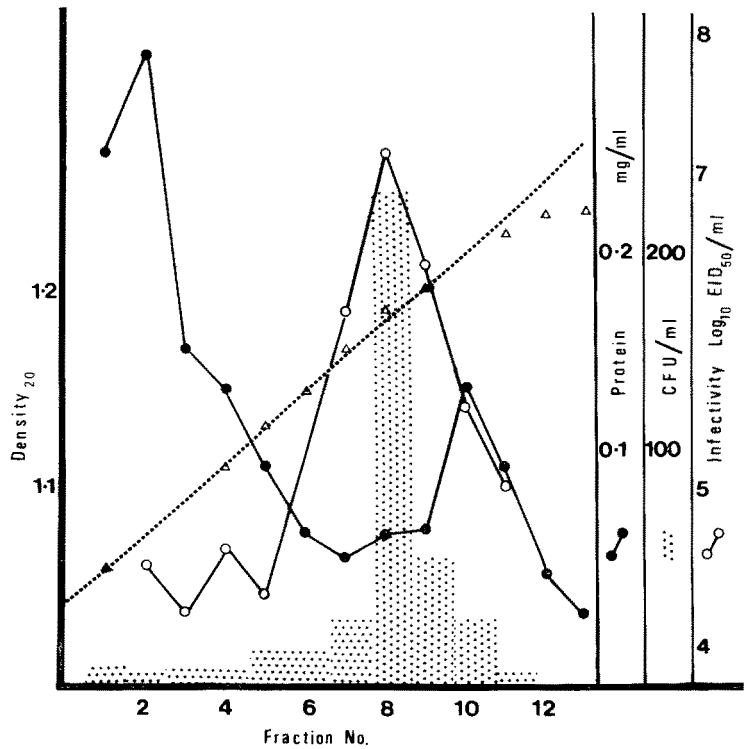


Fig. 1. Isopycnic centrifugation of infectious bronchitis virus
Centrifugation of partially purified IBV on 0—60 per cent sucrose gradients, for 2 hours at 17,000 g. Distributions of density △------△; protein •———•; and infectivity o———o. Complement fixing activity is indicated by the histogram

### *3.2. Sucrose Gradient Analysis of IBV RNA*

Virus labelled with $^{32}P$ and extracted by the phenol-cresol method used by KIRBY (13) revealed a range of RNA sizes after centrifugation on 15—30 per cent sucrose gradients (data not shown) similar to those described by TANNOCK (15). In further experiments virus, disrupted by SDS and analysed by rate zonal centrifugation on 15—30 per cent linear sucrose-SDS gradients, revealed two peaks containing acid-insoluble $^{32}P$. These peaks sedimented with values of approximately 50S and 90S. Some $^{3}H$-leucine activity was associated with the 50S component, but a much larger peak was associated with the 90S component (Fig. 3) probably representing undegraded virus, or ribonucleoprotein.

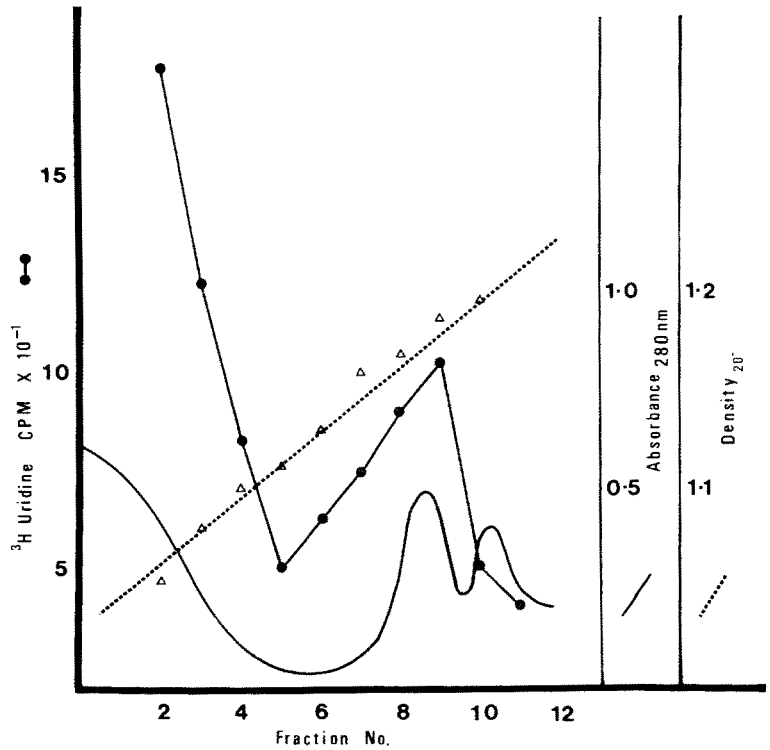


Fig. 2. Uptake of $^{3}H$-uridine associated with IBV
Centrifugation of partially purified $^{3}H$-uridine labelled IBV on 0—60 per cent sucrose gradients, for 2 hours at 17,000 g. Distributions of density △------△; protein concentration (optical density at 280 nm) ———; and $^{3}H$-uridine radioactivity •———•

Parallel experiments with unlabelled Sendai virus also showed a peak of absorbance (measured at 260 nm) which sedimented with a value of approximately 50S. Faster sedimenting material was also observed at the bottom of the gradient and was assumed to be partially disrupted, or whole, virus.

### *3.3. Polyacrylamide Gel Electrophoresis of IBV RNA*

Analysis of SDS-disrupted, $^{32}P$ and $^{3}H$-leucine labelled IBV by electrophoresis on 2.2 per cent polyacrylamide gels revealed a single major radioactive peak with a molecular weight of $9.0 \times 10^6$ (approximately equivalent to 60S) (Fig. 4). In sucrose gradients two peaks of $^{32}P$ activity were seen, whereas PAGE resolved only one band although some activity remained at the top of the gels. The pore size of the gel used in these experiments would have excluded large molecular weight material such as the 90S component seen in sucrose gradients. The apparent discrepancy between the size of the RNA estimated by rate zonal centrifugation and PAGE was examined by analysis of the 50S component from sucrose gradients by PAGE. A peak of molecular weight $9.0 \times 10^6$ (A) was seen but components of lower molecular weight (B and C) were also present (Fig. 5). We consider the components B and C to be degradation products produced during the reisolation of the 50S component from fractions of the rate zonal gradients and not subgenomic fragments as such.

Sensitivity of the 50S component of SDS disrupted IBV to ribonuclease (RNAse) was demonstrated. Part of each fraction from the 15—30 per cent sucrose gradient was precipitated with 99 per cent ethanol at —20° C, after addition of

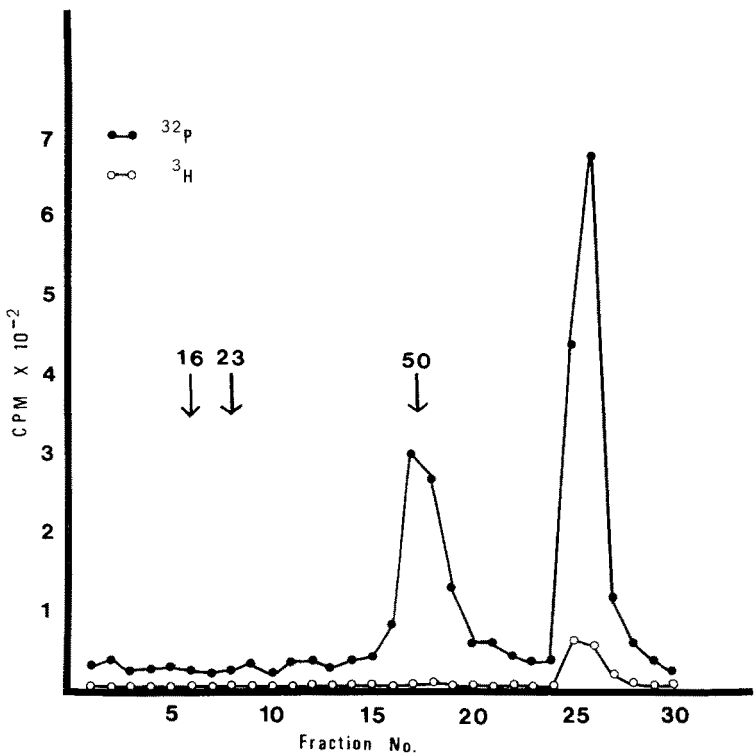


Fig. 3. Rate zonal centrifugation of SDS-treated IBV
Purified $^{32}P/^{3}H$-leucine labelled IBV in 1 per cent SDS was centrifuged on linear 15 to 30 per cent sucrose gradients containing SDS for 16 hours at 15,000 g. TCA insoluble radioactivity: $^{32}P$ •———•; $^{3}H$-leucine o———o

100 μg of carrier RNA. The fractions were washed onto glass fibre circles with cold phosphate buffered saline (PBS) and the filters incubated in 2.0 ml PBS containing 10 μg/ml RNase at 28° C for one hour. The filters and incubation media were then washed with ice-cold 5 per cent w/v TCA and radioactivity determined. The fraction containing the 50S component showed almost complete RNAse sensitivity, yielding only 10 per cent of the original TCA-insoluble activity (from 550 cpm per 0.1 ml to 60 cpm per 0.1 ml).

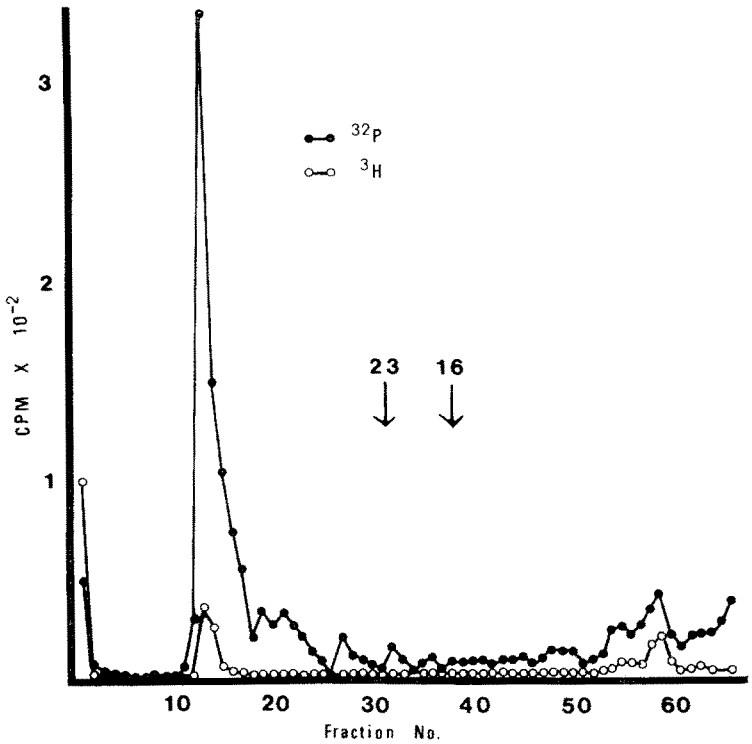


Fig. 4. Polyacrylamide gel electrophoresis of SDS-treated IBV
Purified $^{32}P/^{3}H$-leucine labelled IBV in 1 per cent SDS was analysed on 2.2 per cent polyacrylamide gels. Radioactivity: $^{32}P$ •——•; $^{3}H$-leucine ○——○

## 4. Discussion

TANNOCK (15) using a phenol-SDS RNA extraction technique, examined the RNA of IBV by sucrose gradient and PAGE analysis. His results revealed a large number of heterogeneous molecular weight components which were also obtained in our own studies using a similar technique. However, we have produced evidence, using a method involving minimum manipulation of the virus, that the RNA genome consists of a single strand which migrates with a molecular weight of $9.0 \times 10^6$ on 2.2 per cent polyacrylamide gels or as 50S RNA on sucrose-SDS gradients. The discrepancy in value under the two systems could be accounted for if secondary structure of the RNA is involved. Further analysis of the IBV genome

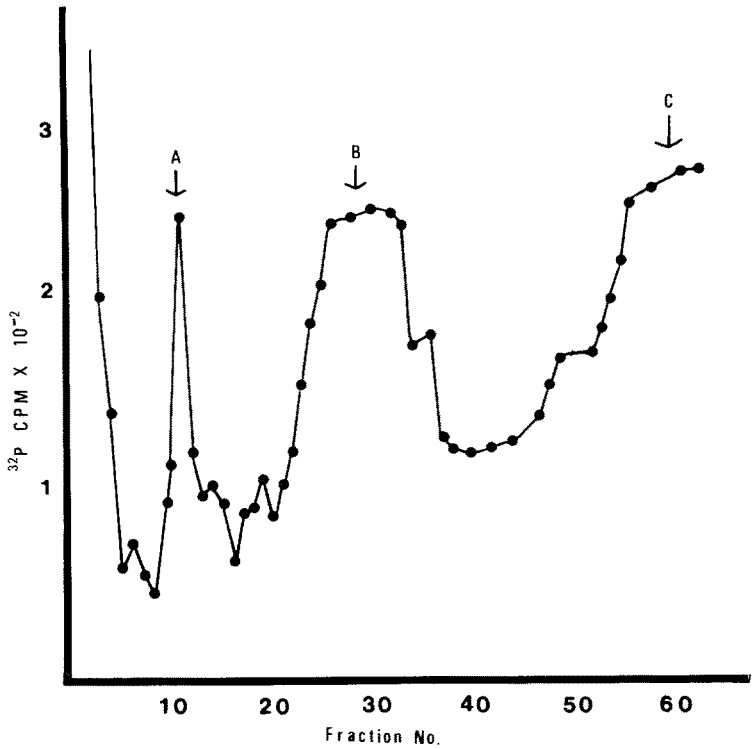


Fig. 5. Polyacrylamide gel electrophoresis of the 50S component from SDS-sucrose gradients

The $^{32}$P 50S component of IBV was precipitated with carrier RNA by 99 per cent ethanol at —20° C and analysed on 2.2 per cent polyacrylamide gels

under more denaturing conditions, such as urea, formaldehyde, or DMSO rate zonal gradients may reveal breaks hidden by secondary structure (6, 10, 12). In studies with Sendai virus (14) partial secondary structure of the RNA genome has been indicated using denaturing DMSO gradients.

Results obtained with double labelled virus do not preclude the possibility that small amounts of protein may be associated with the RNA structure under the conditions employed in this study. Further examination is required to determine the relationship of the RNA to the virion. Whether it exists as a nucleocapsid similar to the paramyxovirus RNA or in some other form is unknown at present.

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