Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Protein Composition of Coronavirus OC 43

J. C. HIERHOLZER, E. L. PALMER, S. G. WHITFIELD, H. S. KAYE AND W. R. DOWDLE

Center for Disease Control, Health Services and Mental Health Administration, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333

Accepted February 2, 1972

A human coronavirus, strain OC 43, was propagated in suckling mouse brain and purified 5000-fold with a 90% yield. Purity of the virus was confirmed by electrophoretic, ultracentrifugal, and electron microscopic procedures. Immunodiffusion and immunoelectrophoresis tests revealed one precipitin line with normal mouse brain, three with purified virus, and four with crude virus when tested against anti-pure virus or anti-crude virus animal serums. The association of a host cell antigen with the virion was confirmed by standard HI and CF tests. Polyacrylamide gel electrophoresis of solubilized purified virus revealed a minimum of six polypeptides with apparent molecular weights of 191,000 (No. 1), 104,000 (No. 2), 60,000 (No. 3), 47,000 (No. 4), 30,000 (No. 5), and 15,000 daltons (No. 6). A seventh band was occasionally found in the 165,000-dalton region of the gels. Four polypeptides contained carbohydrate and one contained lipid. Polypeptide No. 5 comprised 26% of the total viral protein and glycopolypeptide No. 3 comprised 23%. Three other components accounted for most of the remaining protein: polypeptide No. 4 (16%), glycopolypeptide No. 6 (14%), and glycolipopolypeptide No. 1 (13%). Glycopolypeptide No. 2 was 8% of the total protein. Bromelin digestion of the viral projections (spikes) removed glycopolypeptides No. 2 and No. 6. Association of the remaining polypeptides with structural components of the virion is only tentatively postulated. The buoyant density in potassium tartrate of the bromelin-treated virus was 1.15 g/cm³ and of the intact OC 43 virion was 1.18 g/cm³. By analytical ultracentrifugation the corrected sedimentation coefficient ($s_{20, w}$) of the OC 43 virion was determined to be 390 ± 16 S, and the apparent molecular weight ($M_W$) was calculated to be 112 ± 5 X 10⁶ daltons.

INTRODUCTION

Coronaviruses, a recently described group of human and animal viruses, resemble myxoviruses in size and structure, although various physical and host-sensitivity characteristics set them apart. These factors have been thoroughly outlined in reviews by Estola (1970) and Bradburne and Tyrrell (1971).

Antigenic characterization of the avian infectious bronchitis virus, the first of the coronaviruses to be described, has revealed three soluble viral antigens in allantoic fluids of infected embryonated eggs (Tevethia and Cunningham, 1968). Similar studies on human coronaviruses have only recently been conducted (McIntosh et al., 1969; Bradburne, 1970; Kaye et al., 1970). In this report we describe the structural protein and antigenic composition of OC 43 virus harvested and purified from suckling mouse brain (SMB).

MATERIALS AND METHODS

Source and purification of virus. OC 43 strain of human coronavirus was grown to high infectivity titers ($10^5 LD_{50}$/0.2 ml) in SMB and harvested as a 20% suspension in 0.01 M phosphate-buffered saline (PBS). The virus was extensively purified by temperature-dependent adsorption to and elution from fresh human “O” erythrocytes and by batch CaHPO₄ chromatography (Kaye et al., 1970). The final purified virus suspen-
sion was concentrated either by ultrafiltration with XM100A or XM300 Diaflo membranes (Amicon Corporation¹, Lexington, MA), by pelleting at 20,000 rpm (26,400g) for 2 hr in a Beckman No. 40 rotor, or by hydrogel extraction (Lyphogel, Gelman Instrument Company, Ann Arbor, MI), depending upon the tests to be performed on the product.

Preparation of immune sera. Antisera to crude virus (20% suspension in PBS), normal SMB (20% in PBS), and purified virus were prepared in adult Swiss white mice, both as serum and ascitic fluid, in adult guinea pigs (Hartley strain), and in adult White Leghorn chickens. For the production of antiserum in mice, five weekly ip injections of 0.5 ml antigen were given, and the mice were exsanguinated 2 weeks after the last injection. For production of ascitic fluid in mice, four weekly ip injections of 0.5 ml antigen were given (Days 0, 7, 14, 21), followed by 0.2 ml of a 10% suspension of sarcoma 180/TG ip on Day 25; 0.5 ml antigen ip on Day 28; paracentesis on Days 36, 39, and 42; and exsanguination on Day 42.

The schedule for producing antiserum in guinea pigs consisted of five weekly sc injections of 2.6 ml of a 50–50 mixture of antigen emulsified in Freund’s incomplete adjuvant; guinea pigs were exsanguinated 2 weeks after the final injection. In chickens a brief schedule was used to enhance the titer of precipitating antibodies. Two milliliters of antigen were given ip and 2 ml of a 50–50 mixture of antigen in Freund’s complete adjuvant were given im in four sites (0.5 ml/site) on Days 0 and 7; the chickens were exsanguinated on Day 13.

Preimmunization serum samples were obtained from all experimental animals, and serum was obtained from control animals to monitor adventitious infections.

Serologic tests. Hemagglutinin (HA) titrations were performed in the microtiter system with 0.01 M PBS diluent, pH 7.2, and 0.5% adult chicken red blood cells (RBC) (Hierholzer et al., 1969). Indirect HA (IHA) tests for incomplete hemagglutinins were performed in the following manner. Serial dilutions of test antigens were mixed in 0.025-ml vol with 0.025 ml of 2 hemagglutinin-inhibition (HI) antibody units of guinea pig anti-pure virus serum and incubated for 2 hr at room temperature. Two HA units of crude virus in 0.025 ml were added and the test reincubated for 1 hr at room temperature. After addition of 0.05 ml of 0.5% chicken RBC suspension and incubation for 1 hr at room temperature, tests were observed for absence of inhibition in wells containing dilutions of test materials.

Complement fixation (CF) tests. CF tests were performed by the standardized microtiter method with overnight fixation of 5 units of complement (Casey, 1965).

Protein measurements. Protein determinations were made by the method of Lowry et al. (1951) and read in a Beckman DB spectrophotometer at 750 nm against a BSA standard curve.

Immunodiffusion (ID). Ouchterlony double-diffusion tests were carried out on 25 X 75-mm glass slides containing 4 ml 0.7% agarose (Kallestad Laboratories, Minneapolis, MN) in 0.01 M PBS, with 0.01% merthiolate as preservative. Wells 3 mm in diameter and 9 mm apart, center-to-center, were filled three times. The plates were incubated at room temperature for 2 days, photographed, stained with amido black (see IE procedure), and rephotographed.

Immunoelectrophoresis (IE). IE was performed on 25 X 75-mm slides containing 4 ml 1% agarose in 0.025 M Tris–barbital–sodium barbital buffer, pH 8.8. Antigen wells 1.5 mm in diameter were filled with 0.02–0.03 ml sample. The samples were electrophoresed for 75 min at 24° and 250 V at 5–5mA/slide in a 0.50 M Tris–barbital–sodium barbital buffer, pH 8.8. Arcs of precipitate developed after application of appropriate antisera to 1-mm troughs and incubation for 1 day at room temperature.

After immunoelectrophoresis the agar slides were stained for protein and protein-bound lipid (Uriel, 1964). The slides were washed for 2 days in 2% NaCl, rinsed for 2 days in distilled water, and dried overnight under filter paper at room temperature. For

¹ Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.
protein staining, the slides were immersed in amido black 10B (0.1% stain in 10% acetic acid and 45% methanol) for 20 min and destained through several rinses in the acetic acid–methanol solvent. For lipids, slides were stained with oil red 0 (saturated solution in 60% ethanol) and destained in 50% ethanol.

**Polyacrylamide gel electrophoresis (PAGE).** Polyacrylamide gel electrophoresis was performed based on the method of Holowczak and Joklik (1967). Gels, 6 × 60 mm, were composed of 7.5% acrylamide, 0.20% N,N'-methylenebisacrylamide, 0.1% sodium lauryl sulfate (SLS), 0.0575% N,N,N',N'-tetramethylethylenediamine, and 0.07% ammonium persulfate, in 0.01 M sodium phosphate buffer, pH 7.2. Samples to be electrophoresed were solubilized by incubation for 1 hr at 37°C with equal volumes of the 0.01 M phosphate buffer containing 4% SLS, 2% 2-mercaptoethanol (ME), and 1.0 M urea. Samples were then heated at 100°C for 1 min. The materials were dialyzed overnight at room temperature against the phosphate buffer containing 0.1% SLS, 0.1% ME, and 0.05 M urea. Samples thus treated and containing 75–135 μg of protein were stabilized to 10–20% sucrose and applied to the gels. The gels were electrophoresed at 78–86 V and at 3.5 mA per tube at room temperature until a tracking dye, 0.05% bromphenol blue, migrated to 1 mm from the distal end of the gel (about 60 min). The reservoir buffer in both chambers during electrophoresis was 0.01 M sodium phosphate, pH 7.2, with 0.1% SLS.

The gels were removed from the support tubes and stained for protein, protein-bound lipid, and protein-bound carbohydrate. For protein staining, the gels were fixed with 10% trichloroacetic acid (TCA) for 4 hr, stained with 0.05% Coomassie blue in 10% TCA for 8–12 hr, and destained for 3–5 days in 10% TCA (Chrambach et al., 1967). Alternately, gels were stained with 0.1% Coomassie blue in 50% methanol/7.5% acetic acid and power-destained as described by Maizel et al. (1970). For lipid staining, gels were immersed in saturated oil red 0 in 60% ethanol for 2 hr, destained with 50% ethanol for 2 hr, and rehydrated in distilled water (Crowle, 1961). For carbohydrate staining, gels were oxidized with 1% periodic acid in 3% acetic acid for 1 hr, rinsed in distilled water for 1 hr, stained with 0.5% reduced acidified basic fuchsin (Schiff’s reagent) for 45 min, destained in distilled water, and stored in 1% sodium metabisulfite (Clarke, 1964).

All gels were scanned for accurate detection and quantitation of bands with a Gilford model 2410 linear transport coupled to a model 2000 recording spectrophotometer (Gilford Laboratories, Oberlin, OH). Gels stained with Coomassie blue were scanned at 640 nm, those with oil red 0 at the absorption maximum of 492 nm, and those with Schiff’s reagent at the absorption maximum of 534 nm.

Molecular weight determinations of the separated polypeptides were performed by PAGE as described by Shapiro et al. (1967). Solubilized cyt. c, RNAse, hemoglobin, carboxypeptidase A, pepsin (all from Nutritional Biochemicals Company, Cleveland, OH), trypsin, ovalbumin, bovine serum albumin (Schwarz/Mann, Orangeburg, NY), and fetuin (GIBCO, Grand Island, NY), and nonsolubilized purified human IgG (courtesy of Dr. H. Daugharty, Center for Disease Control) were used as molecular weight standards in coelectrophoresis and companion electrophoresis runs. Fetuin was also used as a carbohydrate standard.

**Enzyme treatment.** Purified virus was treated with bromelin (NBC, Cleveland, OH) in a reducing buffer with dithiothreitol as described by Compans et al. (1970) and was subsequently repurified on a 5–40% potassium tartrate gradient by equilibrium centrifugation at 75,000g.

**Electron microscopy (EM).** Negative contrast electron microscopy was carried out with concentrated samples sprayed onto carbon-coated grids, stained with 2.0% sodium phosphotungstate (PTA), and examined at 80 kV with a Philips EM-300 electron microscope.

**Ultracentrifugal analysis.** Sedimentation coefficients and estimates of molecular weight were determined at 15, 16, and 18,000 rpm and at 20.0°C with a An-H titanium rotor and 12-mm Kel-F cells in a Beckman Model E ultracentrifuge equipped with schlieren,
interference, and uv optical systems. The virus samples were dialyzed against 0.01 M sodium borate buffer, pH 8.0, with 0.16 M NaCl, and contained 1–2 mg protein per 0.4 ml. Apparent molecular weight ($M_W$) was calculated from equations derived by Svedberg and Pedersen (1940) and as applied by Burness and Clothier (1970).

RESULTS

**Virus purity.** The virus used throughout these studies was purified to a 5000-fold decrease in total protein, with a yield of 90%. The purity of the virus (at an HA titer of $1.3 \times 10^3$) was confirmed by immunologic procedures, density gradient centrifugation, electron microscopy, and analytical procedures. Electron microscopy at low and high magnifications showed very clean fields of particles exhibiting the pleomorphism typical of this virus (Almeida and Tyrrell, 1967; Almeida and Waterson, 1970; Apostolov et al., 1970; McIntosh et al., 1970; Bradburne and Tyrrell, 1971). Analytical ultracentrifugation revealed a sharp peak at 15,000 rpm with as little as 1.0 mg protein in the sample; this peak had the same sedimentation rate as one of several broad peaks observed when crude virus (at 2–4 mg protein/4 ml) was sedimented under identical conditions. Acrylamide gels loaded with untreated concentrated supernatant fluids obtained from centrifugation (23,800g, 2 hr) of purified virus suspensions revealed no protein bands.

**Immunization and serology.** Mice, guinea pigs, and chickens were immunized with normal SMB (NSMB), crude SMB-grown OC 43, and purified virus. All sera obtained before and during the course of immunization were tested for HI, CF, and precipitating antibody. Preimmunization serum samples and sera from normal animals were consistently negative. Among the postimmunization serum samples, mouse ascitic fluid and chicken antisera had the lowest HI and CF antibody titers to OC 43 virus and guinea pig antisera had the highest titers (Table 1). Sera from mice and guinea pigs immunized with NSMB showed low-level HI and CF activity against the purified OC 43 antigen.

**Immunodiffusion with unsolubilized virus.** Mouse ascitic fluids and mouse antisera prepared against crude virus and purified virus gave predominantly single bands (of identity) with pure or crude virus in standard ID tests. However, tests with guinea pig and chicken anti-crude and anti-pure virus sera showed two precipitin bands of complete identity between pure and crude virus, and one additional band of identity between pure virus, crude virus, and NSM (Fig. 1A). These results suggest that the OC 43 virion contains at least one mouse brain antigen. Additional evidence of host antigen in the intact virus is seen by the identical line between anti-NSMB serum and the three concentrated antigens (Fig. 1B). Immune-electrophoresis of these same antigen-antiserum combinations demonstrated three or four precipitin arcs with crude virus and two–three arcs with purified virus, and con

| Immunizing antigen | Mouse asctic fluid | Guinea pig | Chicken |
|--------------------|--------------------|------------|---------|
|                    | HI     | CF    | HI     | CF    | HI     | CF    |
| Normal SMB         | 20     | 8     | <10    | <8    | 20     | 8     |
| Crude virus        | 1280   | 128   | 320    | 32    | 5120   | 512   |
| Purified virus     | 1280   | 256   | 160    | 32    | 2560   | 1024  |

a All titers listed represent the average antibody titer of five animals per immunizing antigen. All tests performed with purified virus antigen.

b Hemagglutination-inhibition titer expressed as the reciprocal of the end point serum dilution.

c Complement-fixation titer expressed as the optimal dilution of serum as determined by the optimal dilution of antigen in a block titration.
confirmed the presence of a mouse brain protein associated with the purified virus (Fig. 2).

PAGE with solubilized virus. The purified virus was solubilized with SLS–ME–urea, and the resulting proteins were separated by PAGE. The staining patterns with Coomassie blue revealed a minimum of six bands, five of which might be considered major (Fig. 3). One additional very faint band was occasionally observed between bands one and two.

The molecular weights of the viral proteins, as determined by co- and companion electrophoresis with proteins of known molecular weights, were estimated to be 191,000; 104,000; 60,000; 47,000; 30,000; and 15,000 in decreasing order (Fig. 4). The very faint band was located in the 165,000-dalton region of several gels. Gels were also stained for lipid and carbohydrate (Fig. 5).

Polypeptide No. 5 (26%) and glycopoly-peptide No. 3 (23%) constituted nearly one-half of the total virus protein. Polypeptide No. 4 (16%), glycopoly-peptide No. 6 (14%), and glycopolypoly-peptide No. 1 (13%) were similar in concentration and comprised most of the remaining viral protein. The lipid content of glycopolypoly-peptide No. 1 was more evident in gels in which the sample was not heated, than in those in which it was heated at 100°C for 1 min. Glycopoly-peptide No. 2 (8%) was found in the smallest quantity (Table 2).

Composition of the projection antigen. The spikes on the virion surface have previously been associated with HA activity and are thought to comprise the major antigens measured by both the HI and CF tests. Hence these surface spikes or "projection antigens," unlike influenza virus, appear to consist of a single species (Kaye et al., 1970). Absence of neuraminidase was confirmed using a highly sensitive assay system (Laver and Kilbourne, 1966; Laver and Valentine, 1969): overnight incubation of 10⁵ HA units of virus with fetuin failed to yield any detectable sialic acid.

Attempts to determine directly which polypeptides comprise the external antigen were unsuccessful. Bands from acrylamide gels were eluted in PBS, dialyzed, and titrated for direct and indirect hemagglutination. In both tests, the apparent residual SLS adversely affected the erythrocytes.

Repeated attempts to determine the composition of the projection antigen by selectively removing the entire projection from the virus envelope also were of limited value. The agents used represented the major chemical groups often employed in similar studies: reducing agents (6.0 M guanidine + 5.0 mM di-thiothreitol (DTT); 0.003 and 0.03 M DTT; 0.005, 0.05, and 0.5 M 2-mercaptoethylamine); oxidizing agents (67.0 and 85.7% dimethylsulfoxide; 0.1 and 0.2% β-propiolactone); ionic detergents (0.1, 0.5, 1.0, and 2.0% deoxycholate; 0.5% Tween 20); and nonionic detergents (1.0% Nonidet P-40; 1.0% P-40 + 0.5 M urea). These chemical treatments, all used in selected buffer, salt, pH, and temperature environments, were uniformly nonselective and were overly destructive to the entire virion.

The bromelin procedure described by
Fig. 3. Protein components of OC 43 virus separated by polyacrylamide gel electrophoresis.

Fig. 4. Molecular weight of OC 43 virus proteins as determined by acrylamide gel electrophoresis.

Compans et al. (1970) for influenza viruses was employed as an indirect means of locating the proteins comprising the projection antigen. The effect of bromelin treatment on purified virus was monitored every 30 min under the electron microscope. The projections were completely degraded after 2 hr of incubation at 37°C with 0.13% bromelin.
The 2-hr test samples, along with the appropriate virus and enzyme controls, were repurified on 5–40% aqueous neutral potassium tartrate gradients in an SW39L rotor at 75,000g for 8 hr. The untreated control virus banded in the 1.18 g/cm³ region of the gradient and was associated with a sharp HA peak. By EM this band was found to consist of typical virions (Fig. 6A, B). The bromelintreated virus banded in the 1.15 g/cm³ region of the gradient; by EM this band contained completely “despiked” but fully enveloped virus particles (Fig. 6C–E). All biological activity was lost by the enzyme treatment (Table 3). The repurified virus control, the purified “despiked” particles, and the comparable area from the gradient purification of the bromelin control sample were examined by PAGE. Polypeptides 2 and 6—both glycoproteins—were absent in the bromelin-treated sample (Fig. 7).

Sedimentation coefficient and molecular weight of virion. Analytical ultracentrifugation of intact, purified virus (HA titer $2.6 \times 10^5$) at 15, 16, and 18,000 rpm revealed a single distinct peak with a sedimentation coefficient ($S_{20,W}$) of $368 \pm 14 \times 10^{-13}$ sec. Corrections for solvent density and viscosity and for partial specific volume of the virus resulted in an $S_{20,W}$ value of $380 \pm 15$ S. The virus concentration was extrapolated to infinite dilution from the data of Burness and Clothier (1970) to give a fully corrected sedimentation coefficient ($S_{20,W}$) of approximately $390 \pm 16$ S. The apparent molecular weight ($MW_s$) was calculated to be $112 \pm 5 \times 10^6$ daltons, so that the particle
TABLE 2

| Polypeptide band No. | Approximate MW (× 10^3)* | Percent of total protein | Staining reactions               |
|----------------------|--------------------------|--------------------------|----------------------------------|
| 1                    | 191                      | 13                       | Lipid, carbohydrate, protein    |
| 2                    | 104                      | 8                        | Carbohydrate, protein            |
| 3                    | 60                       | 23                       | Carbohydrate, protein            |
| 4                    | 47                       | 16                       | Protein                          |
| 5                    | 30                       | 26                       | Protein                          |
| 6                    | 15                       | 14                       | Carbohydrate, protein            |

* Mean of a minimum of 38 gels from 14 electrophoresis runs on seven different lots of purified virus.

weight of one OC 43 virion is approximately 18 ± 1 × 10^{-17} g.

DISCUSSION

The coronavirus virion has been the subject of a number of studies during the past few years and much is now known about its morphology. The coronaviruses contain RNA (Hamre et al., 1967; Becker et al., 1967; Bradburne and Tyrrell, 1971) which may be present as a form of ribonucleoprotein (RNP) or internal nucleocapsid. Tevethia and Cunningham (1968) thought that “antigen 2” of avian infectious bronchitis (AIB) virus was RNP since it was sensitive to both ribonuclease and proteolytic enzymes. Becker et al., (1967) described an “inner shell” which may have been an internal nucleocapsid. More positive proof is offered by Apostolov et al. (1970) who found in AIB virus a threadlike internal component 7–8 nm in diameter.

Most reports agree that coronaviruses possess an outer double membrane and an inner layer (Becker et al., 1967; Apostolov et al., 1970; Uppal and Chu, 1970; Oshiro et al., 1971). For AIB and 229E viruses, Becker et al. (1967) described a 7–8-nm thick outer shell, a 4–8 nm electronlucent zone, and a 9–17-nm thick inner shell. Oshiro et al. (1971) found a similar pattern in the Linder strain of 229E. Apostolov et al. (1970) describe this envelope as a “3-layered unit membrane structure.” The viral envelope of AIB and 229E appears to contain host lipoprotein (Becker et al., 1967; Berry and Almeida, 1968, Apostolov et al., 1970). This finding is consistent with the mode of virus reproduction—budding from intracytoplasmic cisternae (Becker et al., 1967; Hamre et al., 1967; Apostolov et al., 1970; Oshiro et al., 1971).

The projections of coronaviruses are approximately 10 nm across the club or petal-shaped terminus and are 15- to 20-nm long (Almeida and Tyrrell, 1967; Bradburne and Tyrrell, 1971). They may be composed of two antigens (Tevethia and Cunningham, 1968).

The number of antigens associated with the coronaviruses is still not clear. Part of the problem may relate to the inability to effectively disrupt the virus. Two–three bands were seen in ID tests and three–four precipitin arcs were seen in IE tests with crude OC 43 virus and ant crude or anti-pure virus sera. These were only observed with highly concentrated antigen preparations. The addition of SLS or of Na N-lauroylsarcosinate in various concentrations failed to increase the number of precipitin lines. Our findings support the multiplicity of bands observed in other studies (Tevethia and Cunningham, 1968; McIntosh et al., 1969, Bradburne, 1970).

The one arc found at the antigen well in ID and IE tests was probably whole virus. Another precipitin line was always continuous with the NSMB material and was, therefore, host related. The host-related antigen may correspond to the anti-host activity measured in HI and CF tests. This is consistent with the observation of Berry and Almeida (1968) that AIB virus contains host cell lipoprotein in the envelope. The additional one or two bands seen, especially
FIG. 6. Electron micrographs of OC 43 virus from control preparations showing typical pleomorphism and corona of surface projections (A, ×50,000; B, ×225,600), and from bromelin-treated preparations showing the complete absence of spikes (C, ×192,600; D, ×112,000) and the intact but weakened envelope (E, ×139,400).
### TABLE 3
**PROPERTIES OF BROMELIN-TREATED OC 43 VIRUS**

| Samplea | Buoyant density (g/cm³) | Infectivity in SMB (log10 LD₅₀/0.02 ml) | Antigen titerb | No. of bands in PAGE | No. of precipitin arcs in IE |
|---------|-------------------------|----------------------------------------|----------------|----------------------|-----------------------------|
| 1 Control virus | 1.18 | 9.8 | 32,768 | 128 | 6 | 3 |
| 2 Bromelin-treated virus | 1.15 | 0.7 | <1 | <2 | 4 | 1 |
| 3 Bromelin control | — | 0.0 | <1 | <2 | 0 | 0 |

* a Harvests from potassium tartrate gradients after equilibrium centrifugation at 75,000g for 8 hr; No. 1 was purified virus incubated for 2 hr at 37°C with 0.1 M Tris-HCl buffer, pH 7.2, containing 0.001 M EDTA and 0.005 M DTT (final concentrations), and collected from the 1.18-1.19 g/cm³ region of the gradient; No. 2 was purified virus treated with 0.13% bromelin in the above buffered medium at the same conditions, and collected from 1.15 to 1.16 g/cm³ region of the gradient; and No. 3 was the equivalent amount of bromelin and buffered medium incubated with PBS, and collected from a gradient in a manner identical to sample No. 2.

* b Titer expressed as reciprocal of end point dilution.

---

**FIG. 7.** Polyacrylamide gel electrophoresis patterns of purified virus treated with 0.13% bromelin for 2 hr and repurified on a tartrate gradient (A) and of the control preparation from the same experiment (B).

In IE tests, probably were the results of electrophoretic breakdown of the virus spikes; indeed, IE tests performed with bromelin-treated (despiked) particles did not exhibit these lines. In these tests only a single arc was developed, and this represented a structure with considerably greater electrophoretic mobility than was exhibited by whole virus. Such observations suggest the antigens reactive in the ID or IE tests are probably associated with the virion surface. At least one or two additional precipitin lines might be expected under conditions of complete virus disruption and release of internal components. Indeed this would be suspected by our studies with PAGE.

A minimum of six polypeptide components were demonstrated under the described conditions of solubilizing and examining purified virus by PAGE. These components ranged in molecular weight from 15,000 to 191,000 daltons. However, these molecular weight
figures can only be considered as approximations since the reliability of the PAGE procedure when used for molecular weight determinations is greatest for proteins in the range of 15,000–165,000 MW and since several sources of error are inherent in the PAGE technique. The six or possibly seven bands found in acrylamide gels most likely represented distinct polypeptides and not oligomers because the inclusion of 1.0 M urea as a hydrogen bond-breaking agent in the solubilizing mixture and the use of heat at 100° would minimize protein aggregation.

The classification of the polypeptides as glyco- or glycolipoproteins must be considered as tentative at this point, since the chemical nature of these complexes cannot be precisely defined by differential staining techniques. The use of more sensitive procedures to confirm the nature of these polypeptides awaits the successful adaptation of OC 43 coronavirus to growth in tissue culture systems.

By indirect methods several of these polypeptides can be related to virus structural components. Glycopolypeptides No. 2 (MW 104,000) and No. 6 (MW 15,000) which together constituted 22% of virus protein were associated with the surface projections. Because of its lipid content, one might speculate that the high molecular weight glycolipopolypeptide (No. 1) representing 13% of the total protein may be the outermost layer of the three-layered envelope.

The remaining polypeptides are not readily localized. Glycopolypeptide No. 3, representing 23% of the total virus protein, polypeptide No. 5, representing 26%, and polypeptide No. 4, a relatively low MW (47,000 daltons) protein making up 16% of the protein are still unaccounted for. These proteins may comprise the remaining two layers of the virus membrane and the protein moiety of the RNP, respectively. The faint band (approximately 165,000 daltons) occasionally observed in acrylamide gels could not represent more than 2% of total virus, and may not be structural protein. In any event, the presence of a minimum of six or seven polypeptides in a virus with a molecular weight of 112 million suggests a highly complex structural organization.

REFERENCES

ALMEIDA, J. D., and TYRRELL, D. A. J. (1967). The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture, J. Gen. Virol. 1, 175–178.

ALMEIDA, J. D., and WATERSHAN, A. P. (1970). Some implications of a morphologically oriented classification of viruses. Arch. Gesamte Virolog. 32, 60–72.

APOPOLOV, K., FLEWITT, T. H., and KRANZL, A. P. (1970). Morphology of influenza A, B, C, and infectious bronchitis virus (IBV) virions and their replication. In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. Mahy, eds.) pp. 3–26. Academic Press, London.

BECKER, W. B., MCINTOSH, K., DEEG, J. H., and CHANOCK, R. M. (1967). Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 239E). J. Virol. 1, 1019–1027.

BERRY, D. M., and ALMEIDA, J. D. (1968). The morphological and biological effects of various antisera on avian infectious bronchitis virus. J. Gen. Virol. 3, 97–102.

BRADBURY, A. F. (1970). Antigenic relationships amongst coronaviruses. Arch. Gesamte Virolog. 31, 352–364.

BRADBURY, A. F., and TYRRELL, D. A. J. (1971). Coronavirus of man. Progr. Med. Virol. 13, 373–403.

BURNES, A. T. H., and CLOTHIER, F. W. (1970). Particle weight and other biophysical properties of encephalomyocarditis virus. J. Gen. Virol. 6, 381–393.

CASBY, H. L. (1963), Part II. Adaptation of LBCF method to microtechnique, In "Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test." U.S.P.H.S. Monograph No. 74.

CHRAMBACH, A., REISFELD, R. A., WYCKOFF, M., and ZACCARI, J. (1967). A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. 20, 150–154.

CLARKE, J. T. (1964). Simplified "disc" (polyacrylamide gel) electrophoresis. Anal. Biochem. 20, 428–430.

COMPANS, R. W., KLEIN, H. D., CALIGUIRI, L. A., and CHOPPIN, W. P. (1970). Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycoproteins. Virology 42, 880–889.

CROWLE, A. J. (1961). "Immunodiffusion." pp. 272, 306. Academic Press, New York.

ESTOLA, T. (1970). Coronaviruses, a new group of animal RNA viruses. Avian Dis. 14, 330–336.

HANKE, D., KINDIG, D. A., and MANN, J. (1967).
Growth and intracellular development of a new respiratory virus. J. Virol. 1, 810–816.

Hierholzer, J. C., Suggs, M. T., and Hall, E. C. (1969). Standardized viral hemagglutination and hemagglutination inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18, 824–833.

Holloway, J. A., and Joklik, W. K. (1967). Studies on the structural proteins of vaccinia virus. I. Structural proteins of virions and cores. Virology 33, 717–725.

Kaye, H. S., Hierholzer, J. C., and Dowdle, W. R. (1970). Purification and further characterization of an “IBV-like” virus (coronavirus), Proc. Soc. Exp. Biol. Med. 135, 457–463.

Laver, W. G., and Kilbourne, E. D. (1969). Identification in a recombinant influenza virus of structural proteins derived from both parents. Virology 38, 403–501.

Laver, W. G., and Valentine, R. C. (1969). Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. Virology 38, 105–119.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maizel, J. V., Summers, D. F., and Scharff, M. D. (1970). SDS–acylamide gel electrophoresis and its application to the proteins of poliovirus- and adenovirus-infected human cells. J. Cell. Physiol. 76, 273–288.

McIntosh, K., Bruckova, M., Kapikian, A. Z., Chanock, R. M., and Turner, H. (1970). Studies on new virus isolates recovered in tracheal organ culture. Ann. N. Y. Acad. Sci. 174, 983–989.

McIntosh, K., Kapikian, A. Z., Hardison, K. A., Hartley, J. W., and Chanock, R. M. (1969). Antigenic relationships among the coronaviruses of man and between human and animal coronaviruses. J. Immunol. 102, 1109–1118.

Osorno, L. S., Schierle, J. H., and Lennette, E. H. (1971). Electron microscopic studies of coronavirus. J. Gen. Virol. 12, 161–168.

Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS–polyacrylamide gels. Biochem. Biophys. Res. Commun. 28, 815–820.

Svedberg, T., and Pedersen, K. O., (1940). “The Ultracentrifuge.” Oxford Univ. Press, London.

Tevethia, S. S., and Cunningham, C. H. (1968). Antigenic characterization of infectious bronchitis virus. J. Immunol. 100, 793–798.

Uppal, P. K., and Chu, H. P. (1970). An electron-microscope study of the trachea of the fowl infected with avian infectious bronchitis virus. J. Med. Microbiol. 3, 643–647.

Urteil, J. (1964). The characterization reactions of the protein constituents following electrophoresis or immuno-electrophoresis in agar. In “Immuno-electrophoretic Analysis” (P. Grabar and P. Burtin, eds.) pp. 35–36. Elsevier, Amsterdam.