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Purification and Biophysical Properties of Human Coronavirus 229E

JOHN C. HIERHOLZER

Respiratory Virology Branch, Virology Division, Bureau of Laboratories, Center for Disease Control, Public Health Service, Department of Health, Education and Welfare, Atlanta, Georgia 30333

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Coronavirus 229E was grown to high titers in diploid fibroblast cells under medium containing twice the normal concentrations of amino acids and vitamins. Growth curves showed maximum virus production at multiplicities of infection of 0.1 and 1; maximum titers of intracellular virus occurred at 22-24 hr and of extracellular virus at 26 hr postadsorption. Tube infectivity titers ranged from $10^{9.0}$ - $10^{9.5}$ TCID₅₀/ml and plaque titers from $10^{10.2}$ - $10^{10.9}$ PFU/ml at the time of peak virus production, when no cytopathology was evident. Virus titer dropped rapidly between 26 and 56 hr, coincident with increasing cytopathology. A single precipitin band was observed in immunodiffusion and immunoelectrophoresis between concentrated virus preparations and antiserum to purified 229E. Neuraminidase and hemagglutinin assays were negative. Virus was purified by two procedures: adsorption to and elution from human "O" erythrocytes and CaHPO₄ gel followed by equilibrium sucrose gradient centrifugation, and PEG precipitation followed by equilibrium glycerol/tartrate gradients and rate zonal sucrose or glycerol/tartrate gradients. Final lots of purified virus containing <0.02% of the crude tissue culture proteins had absorption maxima at 256 nm and minima at 241.2 nm and a mean extinction coefficient of $E_{1\%}^{1\text{cm}} = 54.3$ at 256 nm. The fully corrected sedimentation coefficient for the intact virion was $S_{20,w}^{0} = 381$ S. PAGE by different techniques revealed seven polypeptides of mean apparent molecular weights between 16,900 and 196,100. Six contained carbohydrate and one contained lipid. Electropherograms of ³H- and ¹⁴C-labeled virus were identical to those of stained gels. Two glycoproteins constituting 25% of the virion protein were identified by bromelin digestion as the spike proteins. The density in sucrose and in potassium tartrate was 1.18 g/ml for the virion and 1.15 g/ml for the "despiked" particle.

INTRODUCTION

The coronaviruses are a relatively "new" group of mammalian and avian viruses, having been described as a separate group only 8 years ago (Tyrrell, 1968). The group includes strains OC-43, 229E, and 692 of man, all of which cause common colds but are antigenically distinct (Kapikian *et al.*, 1973), possibly other strains associated with gastroenteritis, hepatitis, or nephritis in man (Holmes *et al.*, 1970; Zuckerman *et al.*, 1970; Wright, 1972; Ackermann *et al.*, 1974; Apostolov *et al.*, 1975; Caul and Clark, 1975), neonatal diarrhea virus of calves (NCDV) (Mebus *et al.*, 1973), hemagglutinating encephalomyelitis virus (HEV) and transmissible gastroenteritis

virus (TGE) of piglets, acute enteritis coronavirus 1-71 of neonatal dogs (Keenan *et al.*, 1976), infectious feline peritonitis virus in cats, rat coronavirus (RCV) and sialodacryoadenitis virus (SDAV) of rats, the murine hepatitis viruses (MHV), turkey bluecomb disease virus, and the avian infectious bronchitis viruses (IBV) (Bradburne, 1970; McIntosh, 1974). Few biological or biophysical data are available for most of these viruses, so that, as a group, the coronaviruses are not well understood and cannot be adequately compared with other virus groups.

We described the protein composition of human strain OC-43 in 1972 (Hierholzer *et al.*, 1972). Since then, virus stability studies with OC-43 and 229E have been re-

ported (Bucknall *et al.*, 1972), and some additional data on OC-43 virus density and fragility have been presented (Sheboldov *et al.*, 1973; Pokorny *et al.*, 1975). In this paper the growth characteristics, chemical composition, and biophysical properties of highly purified human coronavirus strain 229E are described.

MATERIALS AND METHODS

Virus culture. Coronavirus 229E was originally provided by Dorothy Hamre, University of Chicago (Hamre and Procknow, 1966), and was obtained for this study from Harold Kaye (CDC, Atlanta) as a throat swab/sHK₂WI38₁₁/RU₈ passage. It was then passaged in RU-1 and HELF human embryonic lung diploid fibroblast strains in Corning 490-cm² plastic roller bottles for production of large quantities of virus. Cells were grown under Eagle's minimal essential medium with 10% fetal calf serum (EMEM₉₀FC₁₀) and maintained at 35° for nonradioactive cultures under EMEM₉₈FC₂ or "fortified" EMEM₉₈FC₂ (Eagle's with twice the normal concentrations of amino acids and vitamins). All media contained 0.07% bicarbonate and 50 µg/ml of chlortetracycline (Aureomycin). When used, actinomycin-D (AMD) ("Dactin," a gift from Merck, Sharpe & Dohme, Rahway, N.J.) was added to fortified EMEM at final concentrations of 0.5–5.0 µg/ml.

Radioactive virus. The virus was internally labeled for some studies with appropriate labels in a fortified maintenance medium consisting of Earle's base, EMEM with 2× vitamins but with no glucose and with only 1/10 the normal concentrations of amino acids, 0.07% bicarbonate, 50 µg/ml of chlortetracycline, and 1% fetal calf serum that had been heat-inactivated at 60° for 2 hr, dialyzed extensively against EBSS, and 0.22 µm-filtered. Labels were L-[³H]amino acids added to 15 µCi/ml final concentration, 1 mCi/mg of D-[¹⁴C]glucosamine at 0.3 µCi/ml, or ¹⁴C-labeled L-amino acids at 1 µCi/ml.

Biological tests. Hemagglutination (HA) tests with a variety of 0.4% mammalian and 0.5% avian erythrocyte suspensions were carried out in a 0.01 M phos-

phate-buffered saline (PBS) diluent according to standardized procedures (Hierholzer and Suggs, 1969). Indirect hemagglutination (IHA) tests for 229E were performed as described by Kaye *et al.* (1972), using block titrations of antigen versus antiserum absorbed with sheep erythrocytes. Complement-fixation (CF) tests were carried out by the standardized microtiter procedure (Casey, 1965). Immunodiffusion (ID) and immunoelectrophoresis (IE) tests were set up with 1% Sea-Kem agarose (Marine Colloids, Inc., Rockland, Maine) in Tris-barbital buffer, pH 8.8, as described (Hierholzer *et al.*, 1972). Samples were electrophoresed for 1.5 hr at 24° and 250 V at 5–8 mA/slide in 0.05 M Tris-barbital buffer, pH 8.8. Antiserum to purified 229E was prepared by immunizing New Zealand White rabbits with three bi-weekly subcutaneous injections of a 50:50 mixture of virus emulsified in Freund's Incomplete Adjuvant, followed by exsanguination 2 weeks after the last injection.

Assays for infectious virus consisted of serial tenfold dilutions of virus, with 0.1 ml inoculated into each of four tubes of HELF without medium, adsorbed for 2 hr at 35°; the monolayers were then covered with 1 ml of fortified maintenance medium. The titrations were read for cytopathology (CPE) at 4, 7, 10, and 14 days of incubation at 35°. Plaque infectivity titrations were performed in HELF monolayers in 8- × 50-mm Falcon dishes as described by Esposito *et al.* (1974), except that fortified maintenance medium was used and the plaques were counted at 7 days.

Acid, chloroform, heat, heat with cationic stabilization, and IUDR stability tests were performed in HELF cells as described (Hierholzer *et al.*, 1975). HELF microcultures infected with 229E were stained with acridine orange and with inclusion body stains as described (Hierholzer *et al.*, 1975). Electron microscopy was carried out without additional concentration steps as described (Hierholzer *et al.*, 1975). Protein assays were made by the method of Lowry *et al.* (1951) with BSA standards. Neuraminidase was assayed as described by Laver and Kilbourne (1966) and by the more sensitive coupled-enzyme system of

Ziegler and Hutchinson (1972).

Purification schemes. (1). This scheme included the purification procedure previously described for OC-43: adsorption of virus to human "O" erythrocytes at 0°, elution in PBS at 37°, followed by adsorption of virus to batch CaHPO₄ gel and elution with 0.3 M phosphate buffer (Kaye *et al.*, 1970). The virus was concentrated and desalted by ultrafiltration at 0° with Diaflo XM-300 Amicon membranes, applied to 20–60% sucrose gradients in PBS, and centrifuged to equilibrium in a Spinco SW-36 rotor at 25,000 rpm for 16 hr. The gradients were fractionated and assayed for infectivity, IHA or CF activity, and density by refractive index.

(2) Virus was precipitated with polyethylene glycol-6000 (PEG) by a modification of the technique of Yamamoto *et al.* (1970). Cells from 229E-infected roller bottles were harvested and virus was released by four freeze–thaw cycles. Cell debris was removed at 1100 g for 20 min at 0° and the pellet resuspended in RSB (0.01 M Tris/0.01 M NaCl/0.0015 M MgCl₂, pH 7.6) and saved for a separate extraction (see below). The supernatant fluid was adjusted to pH 7 and raised to 0.5 M salt and to 8.3% PEG concentrations with rapid mixing in an ice bath. The solution was held at 0° for at least 5 hr, then centrifuged in a Spinco JA-14 rotor at 10,000 rpm for 30 min. The pellet was resuspended in NET buffer (0.15 M NaCl/0.005 M EDTA–Na₂/0.02 M Tris, pH 7.2). Clumps were disrupted with a Teflon homogenizer and the homogenate clarified at 5000 rpm. The supernatant was then layered onto "shallow" positive density/negative viscosity, glycerol/tartrate gradients (Obijeski *et al.*, 1974), which were centrifuged in a Spinco SW-41 rotor at 31,000 rpm for 16 hr. Virus bands were collected by aspiration.

Cell-associated virus from the initial clarification step was released by homogenizing and clarifying in a Spinco JS-13 rotor at 1000 rpm for 10 min. The supernatant was layered over glycerol/tartrate gradients as above, and the virus bands were pooled with those from the PEG-precipitated material. The virus was then diluted in NET buffer to a density of 1.08 and

concentrated over a cushion of 95% glycerol in NET buffer by centrifugation in a Spinco SW 25.1 rotor at 25,000 rpm for 4.5 hr. The concentrates were collected and centrifuged through 20–60% sucrose gradients in NET buffer or "steep" glycerol/tartrate gradients in an SW-41 rotor at 31,000 rpm for 16 hr for isopycnic banding or, for most purifications, for 4.5 hr for rate zonal banding. Virus bands were recovered by aspiration and tested for biological activity.

Bromelin digestion. Bromelin treatment of the virus to enzymatically remove the spike proteins was carried out as described by Compans *et al.* (1970).

Absorption spectrum. Absorption analyses of purified virus were carried out with a Beckman DB spectrophotometer from 360 to 220 nm at a 10 nm/min scan speed and a 2.5 cm/min linear volt recorder chart speed. Solvents and reagent blanks were 0.3 M phosphate buffer, pH 7.2, NET buffer, and the PP-NaCl buffer, pH 8.0, described by Burness (1969).

Analytical centrifugation. Ultracentrifugal analyses were carried out as previously described (Hierholzer *et al.*, 1972). For equilibrium runs, the virus was adjusted to a density of 1.18 with a final concentration of 40% sucrose in 0.01 M sodium borate/0.16 M NaCl buffer, pH 8.0, and centrifuged for 22 hr at 39,000 rpm at 20.0°.

Polyacrylamide gel electrophoresis (PAGE). PAGE was carried out by three procedures: (1) a discontinuous Tris buffer system with 60- and 90-mm 8% gels (Laemmli, 1970); (2) a continuous 7.5% gel/phosphate buffer system at pH 7.2 (Hierholzer *et al.*, 1972); and (3) a continuous pH 7 phosphate system with 120-mm 8% gels (Obijeski *et al.*, 1974). Gels were stained for protein in 0.2% Coomassie brilliant blue G-250 (Serva, Heidelberg) in 50% methanol/7.5% acetic acid and power destained in 7.5% acetic acid/5% methanol (Maizel *et al.*, 1970). The gels were then equilibrated against water and scanned at 1 cm/min in a Gilford Model 2520 linear transport coupled to a Model 2000 recording spectrophotometer (Gilford Labs, Oberlin, Ohio) at 640 nm with 0.05-mm

slits and a chart speed of 5 cm/min. Phosphoprotein staining of gels was carried out by the methods of Cutting and Roth (1973); these gels were scanned at the absorption maximum of 628 nm. Gels for carbohydrate staining (Clarke, 1964) and lipid staining (Crowle, 1973) were handled as described previously (Hierholzer *et al.*, 1972) and scanned at their absorption maxima of 541 and 499 nm, respectively.

Molecular weights of the viral polypeptides in gels were estimated by the method of Shapiro *et al.* (1967). Six to ten standard proteins at a sample size of 25 μ g in 0.1 ml were included with each PAGE run. Additionally, α -casein served as a phosphoprotein standard and fetuin as a glycoprotein standard. Percentage composition was determined by measuring the areas under the spectrophotometric scans with a Hruden planimeter. Molecular weight markers for PAGE with radiolabeled 229E were ¹⁴C-labeled VSV-Indiana proteins (Obijeski *et al.*, 1974) provided by J. Obijeski, CDC.

Radioactive counting. Aqueous samples were solubilized and radio-assayed in a scintillation fluid consisting of 3 parts Triton X-100 (New England Nuclear, Boston MA), 6 parts toluene containing 4 g of Omnifluor (New England Nuclear) per liter, and 1 part distilled water. Acrylamide gels were frozen on dry ice and sliced in 0.8- or 1.0-mm sections with a Mickle Gel Slicer; the slices were partially solubilized at 37° overnight and counted in Omnifluor/toluene (4 g/liter) with 4% NCS solubilizer (Amersham/Searle, Chicago, Ill.). Counting was done in a Packard Tri-Carb Model 3375 Scintillation Counter with automatic channel selection and data computation.

RESULTS

Biological Properties

229E was not inhibited by 10⁻⁴ M IUDR or BUDR in 7-day tube titration tests and produced a red cytoplasmic fluorescence in 1-3 days with acridine orange in HELF microcultures, findings consistent with a single-stranded RNA virus. The virus was chloroform-labile (5% CHCl₃, 10 min), heat-labile with or without 1 M MgCl₂

(50°, 1 hr, pH 7.0), and by conventional definition, acid-labile (pH 3, 4 hr, 23°). Cytopathology became evident after 2 days of incubation and appeared as a generalized deterioration of the monolayer, with shrinking of individual cells and a marked granular and stringy appearance to the cell sheet. Detection of inclusion bodies in HELF microcultures with low input multiplicities and stained at 7, 14, and 18 days was minimal. Slides stained with May-Grunwald-Giemsa and with van Orden inclusion stains when the cultures exhibited 1+ CPE (i.e., 25% of the cells were visibly affected) showed marked karyorrhexis, karyolysis, diffuse necrosis of isolated cells, vacuolation of the fibroblast stroma, scattered nuclear debris, and occasional cells with multiple, round to oval, intranuclear, inclusionlike bodies distinguishable from degenerating chromatin.

During an investigation of various maintenance media and cell culture systems, we found that media enriched in vitamins and amino acids resulted in significantly higher infectivity titers. Thus, after 3 days of incubation at 35°, a HELF culture under regular EMEM₉₈FC₂ gave a tube titer of 10^{3.0} TCID₅₀/ml and a plaque titer of 10^{4.2}/ml, whereas an identical culture under fortified EMEM₉₈FC₂ gave a tube titer of 10^{5.5} TCID₅₀/ml and a plaque titer of 10^{7.0}/ml. Growth curve experiments were then initiated to determine the optimal time of harvest. The growth of radiolabeled virus in AMD-treated cells would have been a choice method. However, 229E at multiplicities of infection (m.o.i.) of 1.0 and 1.3 did not replicate in HELF cells in the presence of AMD at any concentration between 0.5 and 4.0 μ g/ml or in RU-1 cells with 1-5 μ g AMD/ml. Lack of replication may be attributable to AMD toxicity which was evident, although minimal, even at 0.5 μ g/ml, the lowest concentration which could adequately prevent replication of the HELF cells. Cells which are more stable in the presence of AMD, such as RD-120 rhabdomyosarcoma, did not support the growth of the virus.

Growth rates were therefore determined at various m.o.i. by infectivity or by yield of radioactive virus at time intervals post-

adsorption, but in the absence of AMD. Preliminary curves showed that m.o.i. of 0.05, 0.025, and 0.01 gave progressively less yield of virus than m.o.i. of 0.1 or 1.0. Full curves were therefore determined for m.o.i. of 0.1, 1.0, and 10. The virus was adsorbed onto 3x-washed and drained HELF monolayers for 2 hr at 35° to give 26 identical tubes at each m.o.i. After adsorption, the monolayers were again washed, covered with 1 ml of labeling medium with [³H]amino acids and unlabeled glucose (1 mg/ml), and incubated at 35°. At various time intervals, two tubes of each m.o.i. were read for CPE and harvested. In harvesting, one tube was frozen at -70° for the "total" yield. From the other tube, the medium was carefully removed to give the extracellular virus yield, and the monolayer was covered with 1 ml of fresh labeling medium (without the label) and frozen to give the intracellular virus yield. All harvests were ultimately frozen and thawed 3x, and titrated in HELF monolayers under fortified maintenance medium.

Peak titers of infective intracellular virus occurred at 22 hr for the 0.1 m.o.i. and at 24 hr for the 1 and 10 m.o.i. curves, and peak titers of total and extracellular virus occurred at 26 hr for all three m.o.i. (Fig. 1). Similarly, peak radioactivity of extracellular ³H-labeled virus that was TCA-precipitated and ethanol washed occurred at 26 hr for all m.o.i. Radioactivity data for TCA-precipitable intracellular and total virus yields, at each time period and for each m.o.i., were similar to the counts from cell controls harvested in identical manner. Cytopathology remained virtually negative during the time of peak virus production and release, but progressed rapidly with concomitant deterioration of infectious virus. It thus appears that the time of harvest is more important than the m.o.i. for achieving high yields of 229E.

Hemagglutination titers at 4 and 37° with crude or purified virus at 10⁹ TCID₅₀/ml were 1:4 with rhesus; and <1:2 with human, vervet, cow, sheep, dog, guinea pig, rat, mouse, gerbil, turkey, goose, and chicken erythrocytes. In block titrations of

virus versus rabbit immune 229E antiserum, virus preparations at 10⁹ TCID₅₀/ml had an IHA titer of 1:1024 against a 1:80 serum titer and a CF titer of 1:64 against a 1:128 optimal dilution of serum.

Immunodiffusion (ID) tests with concentrated crude and purified virus preparations at 10¹⁰ TCID₅₀/ml versus rabbit antiserum and human convalescent sera consistently gave a single heavy band of identity near the antigen well. The convalescent sera from children infected with 229E (Kaye and Dowdle, 1975) showed a fainter band than did the rabbit antiserum. Immunoelectrophoresis (IE) tests with the same reactants gave the same results as did ID tests; in neither test were reactions to host cell proteins detected.

Neuraminidase assays performed by either the sialic acid test or a more sensitive coupled-enzyme procedure were negative with virus preparations at 10¹⁰ TCID₅₀/ml.

Virus Purification

The two purification procedures described in Materials and Methods were equally usable. The six lots of purified virus prepared by the erythrocyte/CaHPO₄ gradient procedure were somewhat cleaner but of lower final infectivity titer than the six lots prepared by PEG/gradient procedures. The first six lots gave a mean 5800-fold reduction in total protein with a 67% yield; the second gave a mean 5100-fold reduction in total protein with a 96% yield.

Criteria of purity were similar to those established for OC-43 (Hierholzer *et al.*, 1972). There were no precipitin lines or arcs in ID or IE tests with purified virus preparations versus antiserum to whole, concentrated, uninfected HELF tissue culture, compared with six lines between crude 229E cultures and anti-HELF serum. There were no protein bands in acrylamide gels loaded with 0.2 ml of supernatant fluid from purified virus preparations after the virus had been pelleted at 24,000 g for 2 hr. This virus-free supernate had a Lowry protein value of < 2 μg/ml. There was no evidence of contaminating proteins by analytical centrifugation. Less than

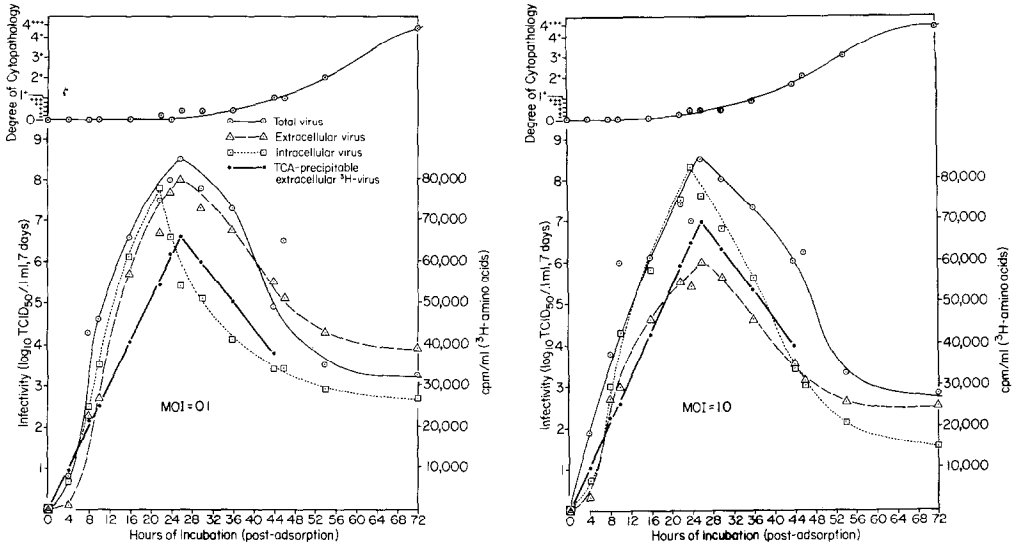


FIG. 1. Growth curves of 229E in HELF cells at m.o.i. = 0.1, 1, and 10. Peak titers of infectious intracellular virus at 22–24 hr were followed by peak titers of infectious extracellular and total virus and by peak levels of ^3H -labeled virus at 26 hr postadsorption, when CPE was minimal. Maximum CPE was coincident with rapid autolysis of the virus as it remained at 35°. CPE was scaled from \pm (5% of cells visibly affected) to +++ (20%) to 1+ (25%) to 4+ (100%) to 4+++ (all cells totally destroyed).

0.007% of total radioactivity remained after purification of radiolabeled virus. Electron microscopy of purified preparations showed clean fields of virions with typical coronavirus morphology and diameters of 78–124 nm (Fig. 2).

Absorption Spectrum

Absorption spectra of different lots of purified 229E at weighed concentrations of 1 mg/ml or less in various simple buffers revealed a mean maximum at 256.0 (range 253–258) nm and a mean minimum at 241.2 (range 241–242) nm (Fig. 3). The mean O.D. 260/280 ratio was 1.53; the specific extinction coefficient, k , for a 1% solution and a 1-cm light path was 51.8_{O.D.,260} and 34.0_{O.D.,280}; the extinction coefficient, $E_{1\text{cm}}^{1\%}$ had a mean value of 54.3 (range 53.3–57.7) at 256 nm. Values were corrected for light scattering by the 360–320 nm baseline subtraction method (Burness, 1970).

Analytical Ultracentrifugation

Sedimentation coefficients for 229E were measured between 15,000 and 19,000 rpm on Schlieren optics. Uncorrected coefficients ($S_{20, \text{b}}^c$) averaged 359×10^{-13} sec;

values corrected for solvent density and viscosity and for partial specific volume of the virus, $S_{20, \text{w}}^c = 377$ S. Virus concentration extrapolated to infinite dilution resulted in a fully corrected sedimentation coefficient, $S_{20, \text{w}}^0 = 381$ S. Equilibrium runs with 229E were of limited meaning due to the rapid disintegration of the virus at 20°.

PAGE

Acrylamide gels were electrophoresed both in discontinuous Tris buffer and in continuous phosphate buffer systems. The electropherograms of stained gels were remarkably similar for the different systems, despite efforts to separate possible overlapping bands by varying gel length and other conditions of electrophoresis. Gels stained for protein, phosphoprotein, glycoprotein, and lipoprotein consistently revealed seven polypeptides: six contained carbohydrate, one contained lipid, but none contained phosphate at a level exceeding 0.6% of the virion by weight (Fig. 4). These polypeptides ranged in estimated molecular weight from the smallest glycoprotein, 16,900 daltons and comprising

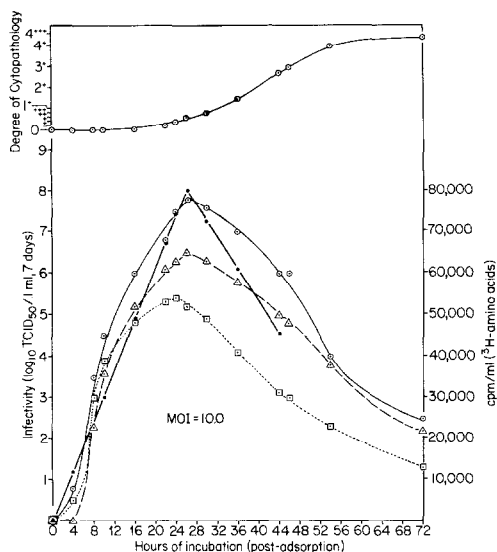


FIG. 1. Continued.

17% of the viral protein, to the lipoglycoprotein, 196,000 daltons and 15% of the viral protein (Table 1).

Two glycoproteins were associated with the surface projections and could be entirely removed from the virion by bromelain: band 3 of 105,500 daltons and band 7 of 16,900 daltons. Together they constituted 25% of the viral protein and a significant part of the virion weight; the density of intact virus in sucrose and in potassium tartrate was 1.18 and of bromelin-treated virus was 1.15 g/ml.

Electropherograms of labeled virus harvested 26–30 hr after adsorption supported both the purity of 229E and the composition of the virus as determined by gels stained for protein and carbohydrate (Fig. 5). ^3H -labeled 229E polypeptides had mean molecular weights of 192,900, 158,600, 107,600, 67,300, 46,900, 31,300, and 17,000, respectively. ^{14}C -labeled carbohydrate was identified in all bands except band 5.

DISCUSSION

Human coronavirus 229E was first isolated in 1962 from a medical student with a mild upper respiratory illness (Hamre and Procknow, 1966). Other than being used as a CF or IHA antigen in a number of respiratory illness sero-surveys, the virus has been studied very little since its discovery

because of its general instability (Bucknall *et al.*, 1972; Pokorny *et al.*, 1975). The virus is clearly IUDR-resistant and chloroform- and heat-labile; it is acid-labile by the definition that it loses at least two logs of infectivity in 4 hr at pH 3.

Growth characteristics of 229E are quite distinct from those of most respiratory viruses. The virus produces at least 2.5 logs more infectious progeny when cultured under a fortified medium containing twice the normal concentrations of amino acids and vitamins than when cultured under unfortified media. Preliminary amino acid analyses on purified virus show that 229E contains five amino acids in concentrations of >8 mol%: aspartic acid, glutamic acid, leucine, serine, and valine. Possibly it is the additional requirement for one or more of these amino acids which results in increased virus yield in fortified cultures.

The virus replicates to equal peak titers with m.o.i. values of 0.1 and 1.0, to slightly lower titers with m.o.i. = 10, and to significantly lower titers with m.o.i. = 0.01–0.05. Highest titers of infectious virus occur at 22–26 hr postadsorption, with no evident CPE during this period. The virus loses titer rapidly as the cultures remain under incubation, with an approximate 0.15 log/hr drop between 26 and 56 hr postadsorption and an abrupt leveling off to 2–3 logs of virus between 56 and 120 hr. The titer drop is concomitant with an increase in CPE, so that the commonly practiced time of harvesting respiratory virus cultures at early 4+ CPE (3–7 days) results in a low-yield passage of 229E. Plaque titers of 229E are 1.2–1.6 logs higher than tube infectivity titers; both titration systems give 26-hr titers of $10^{9.2}$ – $10^{9.6}$ TCID₅₀/ml or $10^{10.4}$ – $10^{10.9}$ PFU/ml.

229E is a relatively poor test antigen and a weak immunogen. The IHA and CF antigen titers are considerably lower than one would expect from nine logs of virus, and the IHA, CF, and SN titers (1:80, 1:128, 1:40, respectively) of antiserum to purified virions also are much lower than expected. Although Bradburne (1970) reported two precipitin lines in ID tests between 229E and human 229E-convalescent sera, we consistently found only one line in

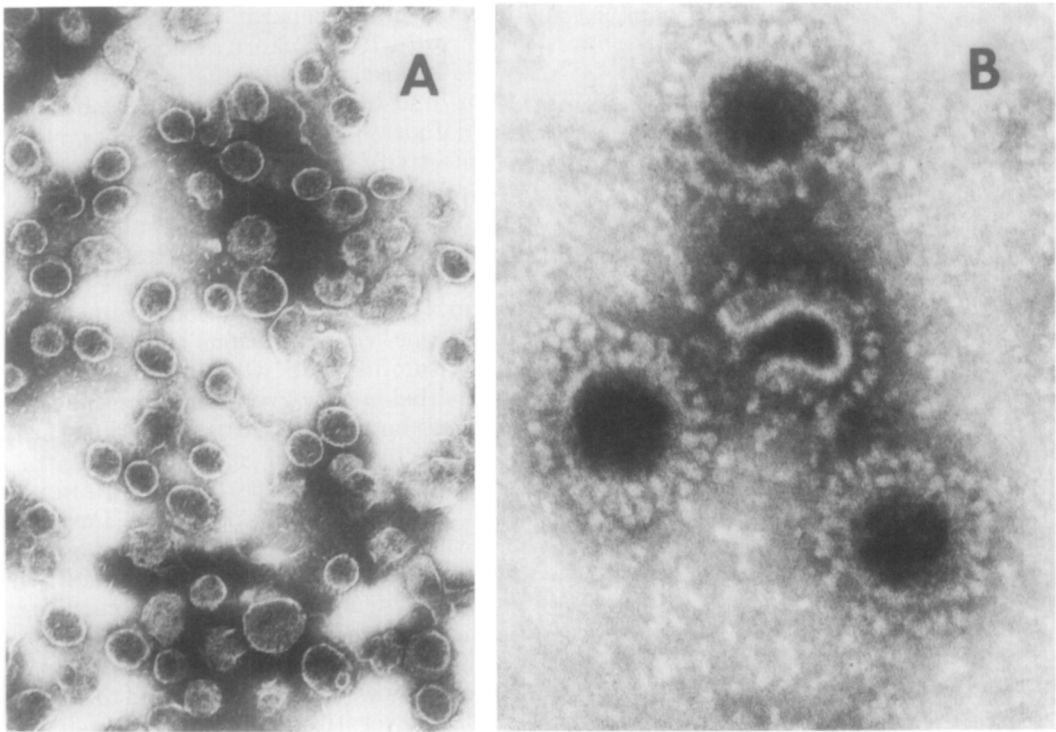


FIG. 2. Electron micrographs of purified 229E as used for analytical studies: (A) 63,400 \times , and (B) 209,600 \times , final magnifications. Grids were prepared by pseudoreplica (A) or spray (B) technique and stained with 2% Na-phosphotungstate at pH 7.

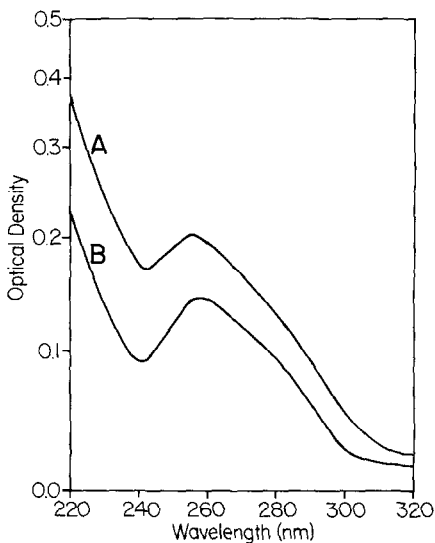


FIG. 3. Absorption spectrums of purified 229E virus: (A) at 350 μg virus/ml in 0.3 M PO_4 buffer, pH 7.2; and (B) at 260 μg virus/ml in NET buffer, pH 7.2. Both scans are with a 1-mm light path.

both ID and IE tests with convalescent human sera and rabbit antiserum versus 10-logarithm preparations of crude or purified virus.

Coronavirus 229E was purified both by an extension of the procedure previously used for OC-43 and by PEG precipitation followed by gradient centrifugations. Both schemes were approximately equal in their removal of host cell proteins, with the final products containing <0.02% of crude tissue culture proteins and <0.007% of crude culture radioactivity, but the PEG system allowed better recovery of intact virus. The fact that 229E could be adsorbed onto human "O" erythrocytes at 0° and then eluted at 37° as the first step in purification scheme (1) was surprising because, unlike OC-43, 229E does not agglutinate these cells. At the same time, OC-43 hemagglutinates by an "adhering" phenomenon (Kaye and Dowdle, 1969), and this

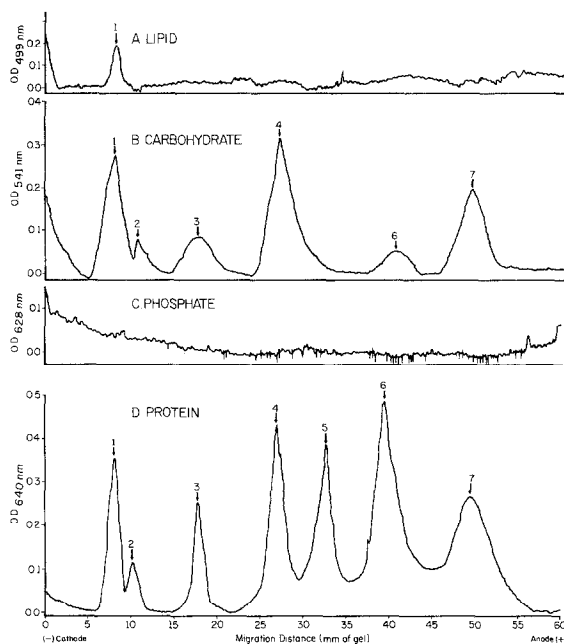


FIG. 4. Spectrophotometric scans of 229E viral proteins separated on discontinuous acrylamide gels and stained for lipoprotein (A), glycoprotein (B), phosphoprotein (C), and protein (D). Gels were electrophoresed in a Tris buffer system with a 3% stacking gel at pH 6.8, an 8% resolving gel at pH 8.8, and an electrode buffer at pH 8.3, at 38 V, 1.5 mA/gel, for 1.2 hr for stacking, and 99 V, 3.0 mA/gel, for 1 hr for resolving.

TABLE 1
PROTEIN COMPOSITION OF CORONAVIRUS 229E

Polypeptide number	Molecular weight ^a		Percentage composition ^b		Staining reactions
	Mean	SD	Mean	SD	
1	196,100	15,800	14.8	4.0	lipid, carbohydrate
2	165,000	15,500	3.0	1.1	carbohydrate
3	105,500	8,900	8.0	2.9	carbohydrate
4	65,500	8,600	20.5	9.9	carbohydrate
5	47,300	6,200	16.5	7.3	—
6	31,400	3,700	20.0	9.0	carbohydrate
7	16,900	2,500	17.2	5.7	carbohydrate

^a Mean of 80 protein-stained gels from 40 PAGE runs on 12 lots of purified virus.

^b Mean of 32 protein-stained gels from 32 PAGE runs on 11 lots of purified virus.

appears to be the manner by which 229E is adsorbed onto human erythrocytes.

Purified 229E has seven polypeptides, the largest of which is a lipoglycopolypeptide and five others of which are glycopolypeptides. No phosphoproteins were detected by the staining procedure used, which in our hands could detect a band with as little as 0.12 μ g phosphate. Spike proteins 3 and 7, both glycoproteins, were

fully removed by bromelain in the same time and under the same conditions required for OC-43 (Hierholzer *et al.*, 1972).

The molecular weights and variation analyses of the polypeptides are means of a large number of gels electrophoresed by various methods and loaded with samples from 12 purification runs. Eight percent resolving gels in the discontinuous Tris buffer system were preferred to gels of

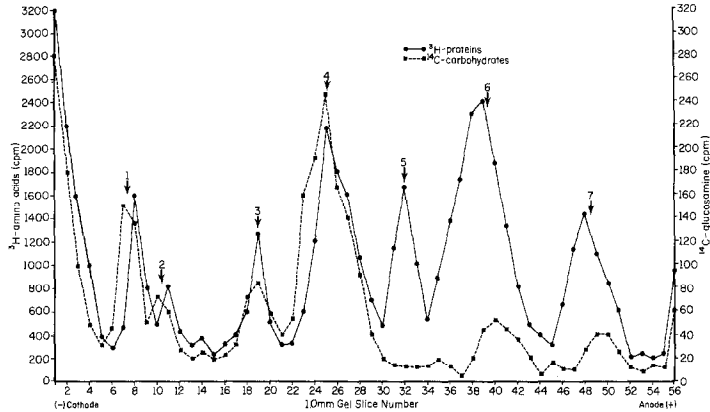


FIG. 5. Radioactive profile of purified 229E virus with ^3H -labeled proteins and ^{14}C -labeled carbohydrates. The viral proteins were electrophoresed in 3%/8% gels in the discontinuous Tris buffer system.

lesser concentration because the molecular weight estimates of glycoproteins are closest to true value in gels of 8% or greater acrylamide concentration (Russ and Polakova, 1973). Also, as observed by many investigators for different viruses, the discontinuous Tris gels gave much sharper bands than did the continuous phosphate gels, although in this study the mean molecular weights and number of bands were the same for the two systems.

The number of polypeptides found in human 229E is compatible with the $4\text{--}5 \times 10^6$ daltons of the major fragments of RNA described for avian IBV by Tannock (1973) and with the 4.4×10^6 dalton RNA observed in OC-43 and 229E (Tannock and Hierholzer, in press). The finding of 16 polypeptides in avian IBV by Bingham (1975) is difficult to imagine with an RNA of this size. Using the assumptions given by Tannock (1973), 16 polypeptides would require an RNA molecular-weight equivalent of approximately 8.5×10^6 . Further work is in progress, however, which hopefully will resolve this discrepancy.

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