

## Pathogenic Murine Coronaviruses

### II. Characterization of Virus-Specific Proteins of Murine Coronaviruses JHMV and A59V

CLIFFORD W. BOND,<sup>1</sup> JULIAN L. LEIBOWITZ, AND JAMES A. ROBB<sup>2</sup>

*Department of Pathology, M-012, University of California, San Diego, La Jolla, California 92093*

*Accepted December 8, 1978*

We have identified nine intracellular virus-specific proteins in cells infected with JHMV or A59V. Seven virus-specific proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two additional virus-specific proteins were detected by two-dimensional gel electrophoresis. The A59V- and JHMV-specific proteins differ slightly in molecular weight. Four of the nine proteins are structural proteins. The synthesis of the nine virus-specific proteins is noncoordinate with respect to time.

#### INTRODUCTION

Several studies of the structural proteins of coronavirus virions have been reported (McNaughton and Madge, 1977; Collins *et al.*, 1976; Bingham, 1975; Sturman, 1977; Sturman and Holmes, 1977; Hierholzer, 1976; Heiholzer *et al.*, 1972). The intracellular virus-specific proteins synthesized in coronavirus-infected cells have not been investigated (Robb and Bond, 1979a). In this report we have identified nine virus-specific polypeptides synthesized in cells infected with either the A59V or JHMV murine coronaviruses. These polypeptides have been identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, radioimmune precipitation, and two-dimensional gel electrophoresis. The time course of virus-specific protein synthesis, the glycosylation of virus-specific proteins, and the identity of A59V and JHMV structural proteins have been studied.

#### MATERIALS AND METHODS

*Cells and media.* Dulbecco-Vogt modified Eagle's medium was supplemented as

described (Robb and Bond, 1979b) and designated DEBO with no serum, DEB2 with 2% fetal bovine serum, and DEB10 with 10% fetal bovine serum. Methionine-free medium was DEB2 without methionine. Amino acid free medium was DEB2 without L-arginine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-serine, L-threonine, L-tyrosine, and L-valine. Glucose-free medium was DEB2 without glucose.

Cell lines used were the 17CL-1 line (Sturman and Takemoto, 1972) and the DBT cell line (Hirano *et al.*, 1974). Cells were grown at 37° in DEB10 in 32-oz prescription bottles.

*Virus infection.* The strains of JHMV and A59V and the titration method are described in the previous paper (Robb and Bond, 1979b). Cells were removed from the glass with 0.1% twice-crystallized trypsin in Puck's saline A supplemented with 0.01 M Tris (pH 8.1) and 0.1% EGTA [ethylene glycol bis-( $\beta$ -aminoethyl ether) *N,N'*-tetraacetic acid], suspended in DEB10, centrifuged at 500 *g* for 2 min, and resuspended in DEB2 at a multiplicity of infection of 0.1 infectious units/ml (iU/ml) at a cell concentration of  $4 \times 10^6$  cells/ml. Adsorption was at 33° for 30 min with agitation at 5-min intervals. The infected cells were sedimented at 500 *g* for 2 min, resuspended in warm DEB2 at a concentration of  $2 \times 10^8$  cells/ml, and 2 ml were

<sup>1</sup> Present address: Department of Microbiology, Montana State University, Bozeman, Mont. 59717.

<sup>2</sup> To whom reprint requests should be addressed at the Department of Pathology, The Green Hospital of Scripps Clinic, La Jolla, Calif. 90237.

plated in a 6-cm plastic dish ( $4 \times 10^6$  cells) or 0.8 ml in a 3.5-cm plastic dish ( $1.6 \times 10^6$  cells). The plates were incubated for the time and temperature indicated in the experiments.

*Radioisotope labeling.* Dishes were removed from the incubator, the media was aspirated, the cells were washed once with warm, nonradioactive labeling medium, 1 ml of labeling medium containing the radioisotope was added to the dish, and the dish was incubated for the time and temperature indicated with each experiment. All radioisotopes were obtained from New England Nuclear.

*SDS-polyacrylamide gel electrophoresis.* At the end of the labeling period, the cells were scraped into the medium with a rubber policeman and centrifuged at 1000 *g* for 30 sec. The medium was removed and the pellet resuspended in 100  $\mu$ l of phosphate-buffered saline (PBS). Ten microliters of  $10 \times$  lysing buffer [600 mM Tris, 320 mM  $H_3PO_4$ , pH 6.7, 10% (w/v) SDS, 1% (v/v) 2-mercaptoethanol] was added and the sample heated at 100° for 3 min. Samples were dialyzed against sample buffer [6 mM Tris, 3.2 mM  $H_3PO_4$ , 0.1 mM  $NaN_3$ , 0.1% (w/v) SDS, 0.1% (v/v) 2-mercaptoethanol].

SDS-polyacrylamide gels were prepared and run as described in Laemmli and Favre (1973) with modifications in the acrylamide-bis ratio as described by Blattler *et al.* (1972). The resolving gels were as described in the experiments with a 5% spacer gel. Equal amounts of protein were applied to each track. The amount of protein in each sample was determined by the method of Bradford (1976) including the precautions suggested by Pierce and Suelter (1977). The radioactivity of each dialyzed sample was determined by drying 5  $\mu$ l on a glass-fiber filter and counting in a liquid scintillation counter. Gels were run for 8 hr at 125 V. The dye marker (phenol red) migrated approximately 18 cm under these conditions.

Molecular weights of proteins were determined by coelectrophoresis of cell lysates with known standards. Unlabeled standards were  $\beta$ -galactosidase (135,000), phosphorylase A (94,000), bovine serum albumin (66,000), catalase (60,000), L-glutamic dehydrogenase (53,000), ovalbumin (43,000), lac-

tic dehydrogenase (36,000), soybean trypsin inhibitor (21,500), myoglobin (17,000), cytochrome C (12,000), crosslinked polymers of bovine serum albumin (66,000, 132,000, 198,000), hemocyanin (70,000, 140,000, 210,000), and hemoglobin (16,000, 32,000, 48,000, 64,000). Labeled standards were intracellular proteins of vesicular stomatitis virus Indiana [190,000, 69,000, 50,000, 45,000, 29,000, (Wagner, 1975)].

*Radioimmune precipitation.* Radioimmune precipitation of whole cell lysates was done essentially by the method of Robb (1977). Infected cells or mock-infected cells were radiolabeled for 20–180 min at various times postinfection with [ $^{35}$ S]methionine. At the end of the labeling period, the cells were scraped into the medium with a rubber policeman, the dish was rinsed with 2 ml of DEB2, pooled and centrifuged at 1000 *g* for 30 sec. The pellet was resuspended in 50  $\mu$ l of 2dA buffer (0.01 *M* Tris-HCl, pH 7.4, 5 mM  $MgCl_2$ , 50  $\mu$ g pancreatic ribonuclease/ml, and 50  $\mu$ g deoxyribonuclease I/ml), sonicated at 80 W peak envelope power for 30 sec, and incubated at 0° for 5 min. The mouse anti-JHMV and anti-A59V antisera were the same as used previously for immunofluorescence (Robb and Bond, 1979). The reaction mixtures consisted of 30  $\mu$ l of cell lysate, 235  $\mu$ l of PBS, 15  $\mu$ l of 10% (v/v) Nonidet-P40 (NP40), and 20  $\mu$ l of mouse anti-JHMV or mouse anti-A59V. The mixture was incubated at room temperature for 90 min, 80  $\mu$ l of goat anti-mouse IgG. IgG was added, and it was incubated at 4° overnight. The radioimmune precipitates were washed twice with PBS containing 0.5% NP40 in a Beckman microfuge, and resuspended in 50  $\mu$ l of  $1 \times$  lysing buffer, heated at 100° for 3 min, and electrophoresed as described above.

*Virus purification.* DBT cells or 17CL-1 cells were infected with JHMV or A59V at multiplicity of infection of 0.1 iU/cell as described above. Following virus adsorption, the cells were sedimented at 500 *g* for 2 min, resuspended in warm DEB2 containing one-fifth the usual concentration of amino acids and 3–5  $\mu$ Ci/ml of a  $^{14}$ C-labeled amino acid mixture (New England Nuclear), plated in 10-cm plastic dishes at  $1 \times 10^7$  cells/dish in 4 ml of medium, and incubated at 33°.

At 16–20 hr postinfection (hpi) when 90% of the cells had formed syncytia the infected cell cultures were subjected to two cycles of freeze–thawing to release cell associated virus. All subsequent operations were performed at 4°. The lysate was clarified at 2500 rpm for 15 min in a PR-2 centrifuge (International Equipment Corporation). The clarified supernatant culture fluids were overlaid onto a 4-ml cushion of 40% (w/w) potassium tartrate in Mops–saline (0.01 M morpholinopropanesulfonic acid, 0.15 M NaCl, pH 6.8) and centrifuged for 1 hr at 25,000 rpm in a SW27 rotor (Spinco). The opalescent band at the top of the cushion was collected by puncturing the tube from the bottom. The virus was diluted with an equal volume of Mops–saline and overlaid onto a 34-ml gradient of 10% (w/w) potassium tartrate and 30% (w/w) glycerol in Mops–saline to 30% (w/w) potassium tartrate and 10% (w/w) glycerol in Mops–saline. Centrifugation was for 5 hr at 25,000 rpm in the SW27 rotor. The gradient was fractionated, aliquots were assayed for radioactivity, and the labeled virus-containing fractions pooled and diluted with 3 vol of Mops–saline. The pooled virus was overlaid onto a second gradient of 10–40% (w/w) potassium tartrate in Mops–saline and centrifuged overnight at 25,000 rpm in the SW27 rotor. The gradient was fractionated and aliquots assayed for acid-insoluble radioactivity. The labeled virus-containing fractions were pooled, the buoyant density determined in a refractometer, and an aliquot assayed for infectivity. Recovery of virus was usually 10–20% by this method.

*Two-dimensional gel electrophoresis.* Samples for two-dimensional electrophoresis were prepared as follows. Cells were radiolabeled as described above. At the end of the labeling period, the cells were scraped into the medium with a rubber policeman, and the dish was rinsed with 2 ml of DEB2, pooled, and centrifuged at 1000 g for 30 sec. The pellet was resuspended in 50  $\mu$ l of 2dA buffer (0.01 M Tris–HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 50  $\mu$ g pancreatic ribonuclease/ml, and 50  $\mu$ g deoxyribonuclease I/ml), sonicated at 80 W peak envelope power for 30 sec, and incubated at 0° for 5 min. Fifty microliters of 2dB buffer [9.5 M urea, 2% (w/v) pH 3–10

ampholines (Bio-Rad), 2% (v/v) NP40, 2% (w/v) sodium deoxycholate, 5% (v/v) 2-mercaptoethanol] was added and the urea concentration was adjusted to 10 M with dry urea.

Two methods of two-dimensional gel electrophoresis were used. Nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension was used to resolve basic proteins (O'Farrell *et al.*, 1977). Isoelectric focusing (IEF) in the first dimension was used to resolve acidic proteins (O'Farrell, 1975; O'Farrell and O'Farrell, 1977). The methods used were modified from those previously described (O'Farrell *et al.*, 1977; O'Farrell and O'Farrell, 1977; O'Farrell, 1975) as follows. The first dimension gels for both methods were poured to a height of 10.5 cm in 13  $\times$  0.25-cm glass tubes. The gel mixture contained 9 M urea, 4.5% (w/v) acrylamide/bisacrylamide [from a 30% (w/v) stock solution consisting of 28.4% acrylamide and 1.6% bisacrylamide], 2% (v/v) NP40, 2% (w/v) ampholines (pH 3–10), 0.03% (v/v) N,N,N',N' - tetramethylethylenediamine (TEMED), and 0.6% (w/v) ammonium persulfate. The gels were overlaid with 8 M urea for 1 hr, aspirated, and overlaid with 20  $\mu$ l of 2dC buffer (8 M urea, 2% pH 3–10 ampholines, 2% NP40) and 40  $\mu$ l of water for 1 hr. The IEF gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. The NEPHGE gels were not prerun. Equal amounts of protein (approximately 40  $\mu$ g) containing approximately 300,000 to 900,000 acid-insoluble counts/min in a volume of 5  $\mu$ l were applied to each gel and overlaid with 20  $\mu$ l of 2dC buffer (8 M urea, 2% pH 3–10 ampholines, 2% NP40) and 40  $\mu$ l of water for 1 h. The IEF gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. The NEPHGE gels were not prerun. Equal amounts of protein (approximately 40  $\mu$ g) containing 300,000 to 900,000 acid insoluble counts/min in a volume of 5  $\mu$ l were applied to each gel and overlaid with 20  $\mu$ l of 2dC buffer. The NEPHGE gels were run in an apparatus with the upper tank (positive electrode) filled with 0.01 M H<sub>3</sub>PO<sub>4</sub> and the lower tank (negative electrode) filled with 0.1 M NaOH for 3.5 hr at 400 V. The IEF gels were run in an apparatus with the upper tank (negative

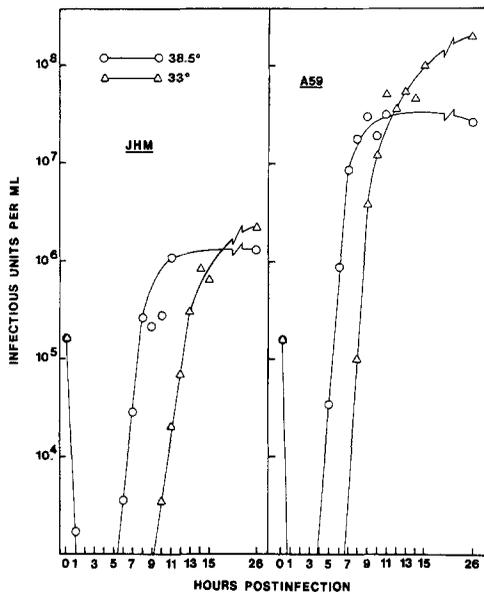


Fig. 1. One-step growth curve of JHMV and A59V in 17CL-1 cells. Cells were infected at an m.o.i. of 0.1 and replicate cultures of  $1.6 \times 10^6$  cells were plated into 35-mm dishes. At the indicated times, dishes were frozen at  $-40^\circ$ , then thawed, sonicated, clarified, and the virus was titered at  $33^\circ$ .

electrode) filled with 0.1 M NaOH and the lower tank (positive electrode) filled with 0.01 M  $H_3PO_4$  for 18 hr at 400 V. At the end of the run, the gels were extruded into sample buffer [0.06 M Tris- $H_3PO_4$ , pH 6.8, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol]. The gels were soaked at room temperature for 30 min, quick-frozen in dry ice-ethanol and stored at  $-70^\circ$ . The gels were thawed, the sample buffer replaced with fresh sample buffer, and soaked an additional 90 min before the second-dimension run. The second-dimension gels were SDS-polyacrylamide slab gels. The resolving gels were 12.5% acrylamide and the spacer gels were 5% acrylamide. The tube gel was laid on top of the spacer gel as described by O'Farrell (1975) and sealed in place with 1% agarose in 0.06 M Tris- $H_3PO_4$ , pH 6.8, 0.1% (w/v) SDS. Electrophoresis was for 8 hr at 125 V. The dye marker migrated approximately 18 cm under these conditions.

**Staining, drying, and fluorography.** Gels containing unlabeled proteins were stained for 2 hr with 0.001% (w/v) Coomassie brilliant blue G in 45% (v/v) methanol,

10% (v/v) acetic acid, and destained in 25% (v/v) ethanol, 8% (v/v) acetic acid. The gels were dried by the method of Studier (1973). Gels were prepared for fluorography by the method of Laskey and Mills (1975) except that the concentration of 2,5-diphenyl-oxazole in dimethyl sulfoxide was reduced to 10% (w/w). The fluorographed gels were exposed to preflashed Kodak XR-2 X-ray film to insure linearity of response (Laskey and Mills, 1975).

## RESULTS

### *Time Course of Virus Multiplication*

The kinetics of JHMV and A59V multiplication at 38.5 and  $33^\circ$  were determined (Fig. 1). These two temperatures were chosen to facilitate later work with temperature-sensitive mutants of JHMV. Both JHMV and A59V gave synchronous infections of 17CL-1 cells using an input multiplicity of 0.1 iU/cell. In cultures infected with JHMV or A59V, the input virus was eclipsed by 1 hr postinfection. Cultures infected with JHMV and incubated at 38.5 or  $33^\circ$  began yielding virus at 6 and 10 hr postinfection and reached their maximum titers at 11 and 26 hr postinfection, respectively. The maximum yield of JHMV with these conditions was approximately  $2 \times 10^6$  iU/ml. The replication of A59V was slightly faster than that observed for JHMV. Cultures infected with A59V and incubated at 38.5 or  $33^\circ$  began yielding virus at 5 and 7 hr postinfection and reached their maximum yield of virus by 9 and 26 hr postinfection, respectively. The data shown in Fig. 1 demonstrates that A59V is somewhat temperature sensitive; the yield of virus from cultures incubated at  $38.5^\circ$  is approximately 10% of that obtained from infected cultures incubated at  $33^\circ$ . The data shown in Fig. 1 are representative of three independent experiments.

### *Time Course of Virus-Specific Protein Synthesis*

The kinetics of virus-specific protein synthesis in cells infected with JHMV was determined (Fig. 2). 17CL-1 cells were infected with JHMV, incubated at 33 and  $38.5^\circ$ , labeled for 1 hr with  $^{14}C$ -labeled amino

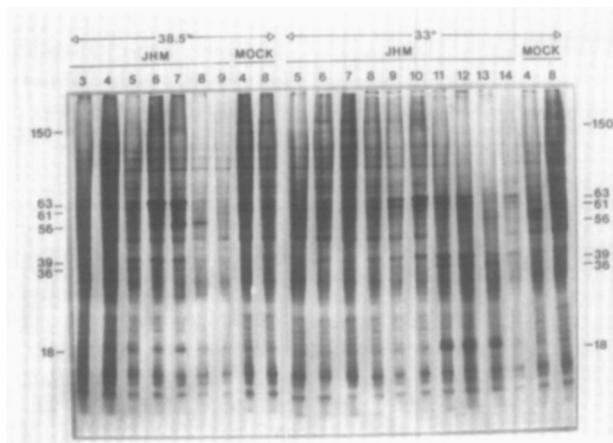


FIG. 2. Time course of JHMV-specific protein synthesis. 17CL-1 cells were infected with JHMV at a m.o.i. = 0.1. The infected cells were plated out in replicate 6-cm dishes and incubated at either 33 or 38.5°. Infected cells ( $4 \times 10^6$  cells/6 cm dish in 1 ml) were labeled with 5  $\mu$ Ci of  $^{14}$ C-labeled amino acid mixture for 1 hr beginning at the time indicated above each gel. Labeling was terminated and total cell lysates were prepared and electrophoresed on 12.5% polyacrylamide slab gels as described under Materials and Methods. The fluorographs were exposed for 2 days.

acids at various times postinfection, and analyzed by SDS-polyacrylamide gel electrophoresis. JHMV does not inhibit host protein synthesis until late in the infectious cycle. Comparison of JHMV-infected cell lysates with mock-infected lysates revealed seven JHMV-specific polypeptides. The positions and apparent molecular weights of these polypeptides are indicated in the figure. The first JHMV-specific protein to be detected was the 150K protein. At 38.5° this protein was detected by 4–5 hpi; at 33° this protein first appeared by 7–8 hpi.

The majority of JHMV-specific proteins were first apparent somewhat later, by 5–6 hpi at 38.5° and 8–9 hpi at 33°. The maximum rate of JHMV-specific protein synthesis was between 6–8 hpi at 38.5° and 10–12 hpi at 33°.

A similar experiment was performed using A59V (Fig. 3). The results were similar to those obtained with JHMV. A59V, like JHMV, does not inhibit host protein synthesis until late in infection. Seven A59V-specific proteins were detected in infected cultures. The positions and apparent

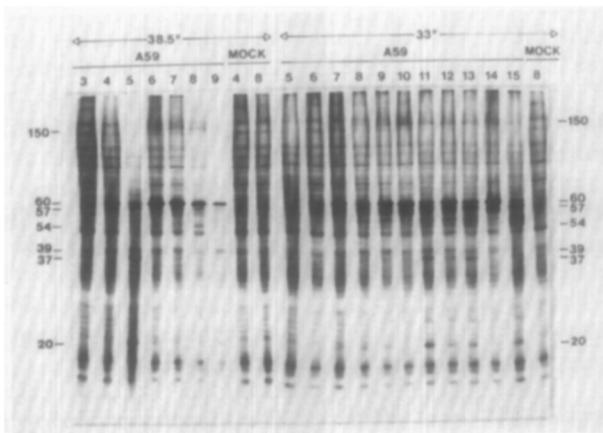


FIG. 3. Time course of A59V virus-specific protein synthesis. Labeling conditions of electrophoresis and exposure were the same as described in Fig. 2.

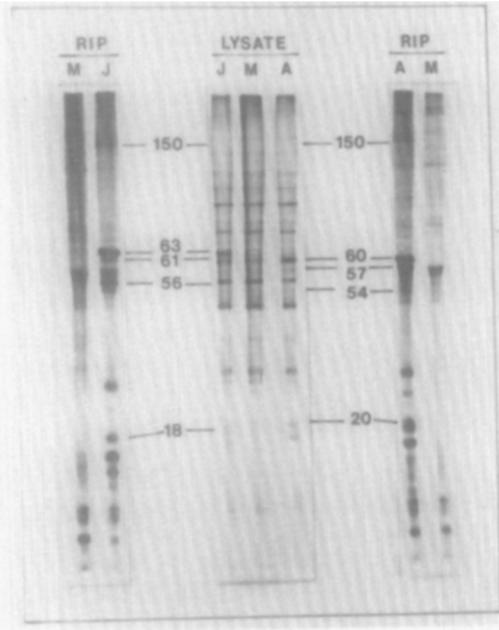


FIG. 4. Coelectrophoresis of radioimmune precipitates and whole cell lysates of cells infected by JHMV or A59V, as mock infected 17CL-1 cells were infected with JHMV or A59V at an m.o.i. = 0.1 or mock infected. The cells were plated in replicate 6-cm dishes and incubated at 38.5°. Cells ( $4 \times 10^6$  cells/6-cm dish in 1 ml) were labeled with 200  $\mu$ Ci of [ $^{35}$ S]-methionine from 7 hr postinfection to 8 hr postinfection. Labeling was terminated and whole cell lysates and radioimmune precipitates were prepared and electrophoresed on 12.5% polyacrylamide slab gels as described under Materials and Methods. The fluorographs were exposed for 1 day.

molecular weights of these proteins are indicated. The molecular weights of the A59V-specific proteins differ slightly from those seen in cultures infected with JHMV. The time course of A59V-specific protein synthesis was similar to that observed for JHMV. The 150K protein was first detected at 4–5 hpi at 38.5° and 6–7 hpi at 33°, 1 hr earlier than the other virus-specific proteins. The maximum rate of A59V-specific protein synthesis is at 6–8 hpi at 38.5° and at 10–12 hpi at 33°. These results have also been confirmed in a series of parallel experiments using DBT cells (data not shown).

#### *Molecular Weights of A59V- and JHMV-Specific Proteins*

The molecular weights of A59V and JHMV-specific proteins were determined

by SDS-polyacrylamide gel electrophoresis. Infected cell lysates were electrophoresed with known molecular weight standards on a series of gels of 5, 7.5, 10, 12.5, 17.5, and 20% acrylamide concentrations. The molecular weights of all proteins other than the 150K protein were determined as described by Maizel (1971). The molecular weight of the 150K protein, a glycoprotein, was calculated according to Segrest and Jackson (1972).

A direct comparison of JHMV- and A59V-infected lysates is known in the center lanes of Fig. 4. The molecular weights of A59V- and JHMV-specific proteins differ slightly with the exception of the 150K and 39K proteins.

#### *Radioimmune Precipitation of Virus-Specific Proteins*

The virus-specific nature of the proteins demonstrated in cells injected with A59V and JHMV was confirmed by radioimmune precipitation using the antisera described and used for immunofluorescence (Robb and Bond, 1979b). The results of one typical experiment are shown in Fig. 4. The lower half of the radioimmune precipitation tracts are distorted due to the high concentration of goat anti-mouse IgG in the samples. The anti-A59V and anti-JHMV antisera markedly enrich for five of the seven proteins shown in Figs. 2 and 3. For this experiment, the cells were labeled for 7 to 8 hpi, and thus the 36K (JHMV), 37K (A59V), and 39K (A59V and JHMV) proteins are not present. At earlier times (5–6 hpi) these proteins are markedly enriched by the antisera. Other proteins, presumably cellular, are precipitated by the antisera in both mock and infected lysates. Three other proteins in the range of 15K and 30K are sometimes found in the radioimmune precipitates of both A59V and JHMV lysates, but not in the mocks (Fig. 4). Since these proteins are not consistently evident in all experiments, we do not consider them to be viral specific.

#### *The Identity of Virion Polypeptides*

DBT cells and 17CL-1 cells were infected with either A59V or JHMV, the virions purified as described under Materials and

Methods, and the purified virions analyzed by one-dimensional polyacrylamide gel electrophoresis (Fig. 5). Four polypeptides are present in A59 virions. These proteins have apparent molecular weights of 150,000, 89,000, 60,000, and 20,000. We have also identified four proteins in JHM virions which differ slightly in molecular weight from the corresponding A59 virion proteins. We also find in JHM virions several polypeptides which are present in small and inconstant amounts. These are probably contaminating host-cell proteins. The electrophoretic mobilities of three of the virion proteins (the 150K, 89K, and 20K A59 proteins and the 150K, 100K, and 18K JHM proteins) are slightly greater with virus

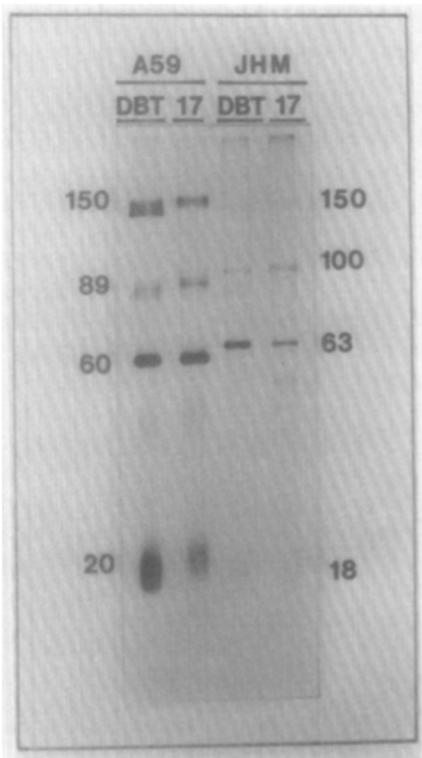


FIG. 5. The polypeptide composition of JHMV and A59V virions. JHMV and A59V virions were labeled with  $^{14}\text{C}$ -labeled amino acids and purified as described under Materials and Methods. Purified virus was diluted with 5 vol of mops-saline, pelleted in the SW50.1 rotor (Spinco) at 45,000 rpm for 1 hr, and resuspended in 100  $\mu\text{l}$  of electrophoresis sample buffer and heated to 100° for 2 min. The samples were electrophoresed on 12.5% polyacrylamide slab gels for 16 hr at 60 V. The gel was prepared for fluorography and exposed for 4 days.

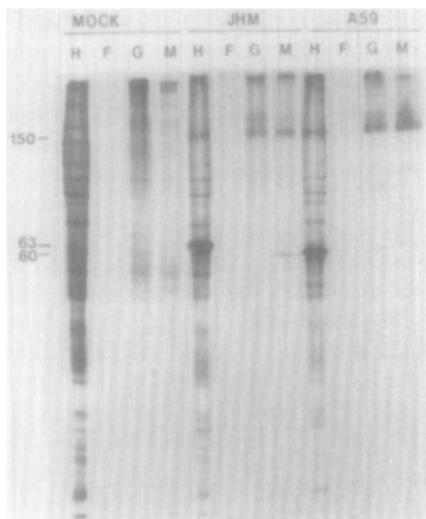


FIG. 6. Intracellular virus-specific glycoproteins of JHMV and A59V at 6.5 hpi. Infected cells (m.o.i. = 0.1,  $4 \times 10^6$  cells/dish in 1 ml) were labeled with 25  $\mu\text{Ci/ml}$  of  $^3\text{H}$ glucosamine (G) or 50  $\mu\text{Ci/ml}$  of  $^3\text{H}$ mannose (M) for 45 min at 38.5°. Conditions of sample preparation, electrophoresis, and exposure are the same as in Fig. 2.

grown in DBT cells when compared to virus grown in 17CL-1 cells. These small, host-cell dependent changes in electrophoretic mobility are consistent with these virion proteins being glycosylated, as has been previously reported (Sturman, 1977; Sturman and Holmes, 1977).

#### *Intracellular Virus-Specific Glycoproteins*

17CL-1 cells were infected with either JHMV or A59V or were mock infected, labeled with radioactive sugars, or amino acids and analyzed by SDS-polyacrylamide gel electrophoresis as described in Fig. 6. No virus-specific protein was detectable with the fucose label. The 150K protein was labeled with glucosamine and mannose in cells infected with either JHMV or A59V. The JHMV-specific protein migrating with an apparent molecular weight of 61,000 and the A59V-specific protein migrating at 60,000 were slightly labeled with mannose. No other virus-specific glycoproteins were detected.

#### *Two-Dimensional Gel Electrophoresis of A59V and JHMV-Infected Cell Lysates*

It was necessary to use  $^{35}\text{S}$ methionine as the label to have cells incorporate suf-

ficient radioactivity into protein for analysis on two-dimensional gels. We compared [ $^{35}\text{S}$ ]methionine and  $^{14}\text{C}$ -labeled amino acid infected cell lysates on SDS-polyacrylamide gels because of the possibility that some virus-specific proteins might not contain methionine. The seven A59V-specific proteins and six of seven JHMV-specific proteins detected on SDS-gels were labeled to approximately the same extent with [ $^{35}\text{S}$ ]methionine as with a  $^{14}\text{C}$ -labeled amino acid mixture. The 18K JHMV-specific protein incorporated less [ $^{35}\text{S}$ ]methionine and was barely detectable on one-dimensional SDS-polyacrylamide gels (data not shown). We elected to determine whether additional virus-specific proteins could be detected by two-dimensional gel electrophoresis because of the high background of host protein synthesis in infected cells. We utilized either IEF or NEPHGE in the first dimension coupled with SDS-polyacrylamide electrophoresis in the second dimension to resolve basic and acidic proteins. Virus-specific proteins were located by comparison with mock-infected cultures (Fig. 9). The two-dimensional gels obtained from a JHMV-infected culture are shown in Fig. 7. The 150K protein was detected only on the gel using IEF in the first dimension for presently unknown reasons. The 63K and 61K proteins were only present on the gel using NEPHGE in the first dimension because these proteins are extremely basic and migrate off IEF gels. The 59K and 39K proteins were detected in gels using either IEF or NEPHGE. The 36K protein is only resolved in gels using NEPHGE as it is probably obscured by the cellular background in IEF gels. The protein labeled E, detected only on IEF gels because of its  $pI$  of 4.1, has an apparent molecular weight of approximately 84,000. This molecular weight corresponds to the molecular weight of a virion structural protein. This protein is also occasionally detected in radioimmune precipitates of JHMV-infected cultures. The protein labeled B has an apparent molecular weight of approximately 75,000 and was only detected on gels using NEPHGE in the first dimension for presently unknown reasons. Protein D has an apparent molecular weight

of approximately 30,000. This protein was not consistently detected and is probably not a JHMV-specific protein. The four spots labeled A, seen in the gel using NEPHGE in the first dimension, have molecular weights of about 24,000–29,000. Most of the spots in this complex did not appear in every experiment. The spot in the lower left-hand side of the A complex was present in multiple experiments and has an apparent molecular weight of approximately 24,000. This spot may correspond to the spot labeled C in the gel using IEF in the first dimension which has an apparent molecular weight of about 20,000.

Similar experiments were performed with A59V. The two-dimensional gels obtained from A59V-infected cultures are shown in Fig. 8. The 150K, 60K, 57K, and 54K proteins are identified by their molecular weights as described for JHMV-specific proteins. The two spots present in the ellipse marked 39 and 37 present a problem. The position of the lower spot corresponds to a molecular weight of 34,000 while the position of the upper spot corresponds to a molecular weight of 37,000. We feel that the upper spot is the 37K A59V-specific protein identified in one-dimensional SDS-gels. We feel that the lower spot is the 39K A59V-specific protein which migrates anomalously in two-dimensional NEPHGE gels. This type of anomalous migration has been reported for certain proteins (O'Farrell *et al.*, 1977). The protein labeled Z, only detected on gels using IEF in the first dimension, has an apparent molecular weight of approximately 89,000. This protein, analogous to the JHMV protein labeled E, probably represents the A59V structural protein of 90,000 molecular weight (Sturman, 1977). The protein labeled X has an apparent molecular weight of 70,000 and corresponds to the JHMV-specific protein labeled B. The spots labeled W and Y both have molecular weights of approximately 20,000 and probably represent the same protein.

The isoelectric points ( $pI$ ) of proteins can be estimated from the lateral position of the protein in the two-dimensional gel when IEF is used in the first dimension. However, when NEPHGE is used in the

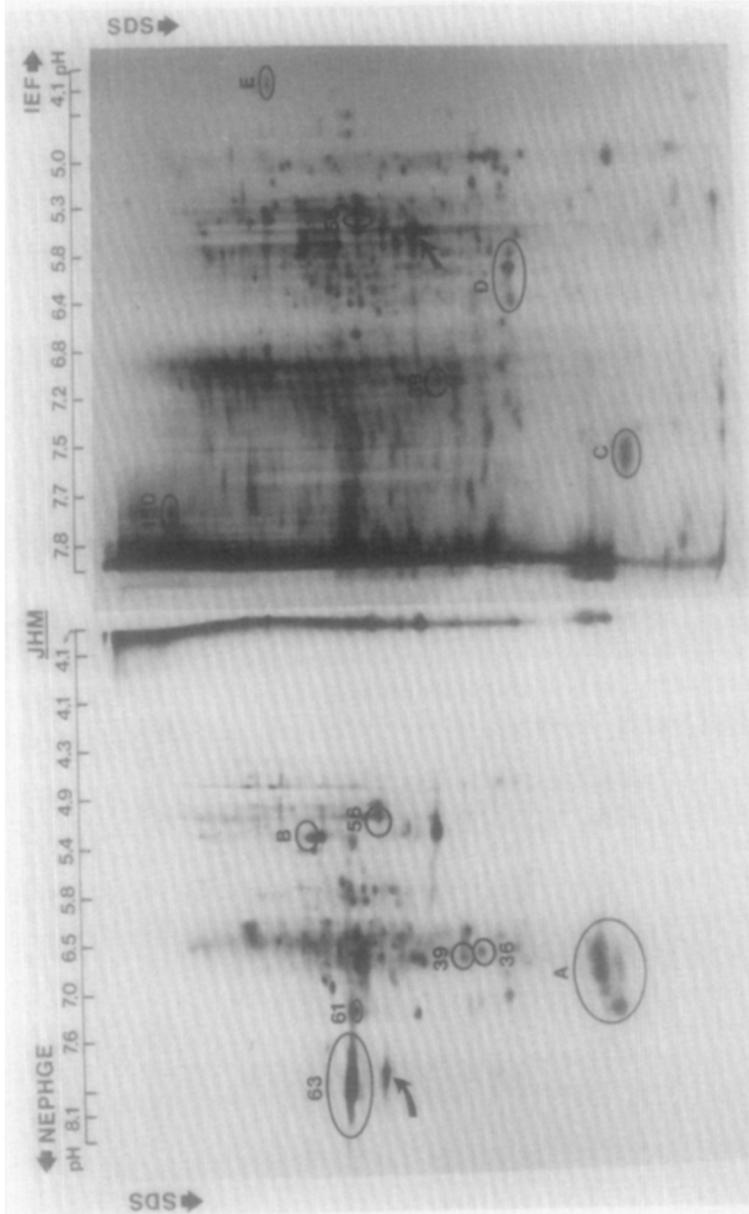


FIG. 7. Two-dimensional electrophoresis of proteins of cells infected by JHMV. JHM-infected cells (m.o.i. = 0.1,  $4 \times 10^6$  cells/6-cm dish in 1 ml) were labeled with [ $^{35}$ S]methionine ( $200 \mu\text{Ci/ml}$ ). Samples were prepared and analyzed by IEF or NEPHGE in the first dimension and by SDS-electrophoresis in the second dimension. The proteins were identified by comparison with the mock profiles (Fig. 9). Examples of cellular proteins are indicated by arrows. Proteins identified as viral-specific proteins are labeled with the molecular weight ( $\times 10^{-3}$ ). Other proteins not present in the mock profiles and not corresponding to viral-specific proteins detected on one-dimensional gels are labeled with letters and are discussed in the text.

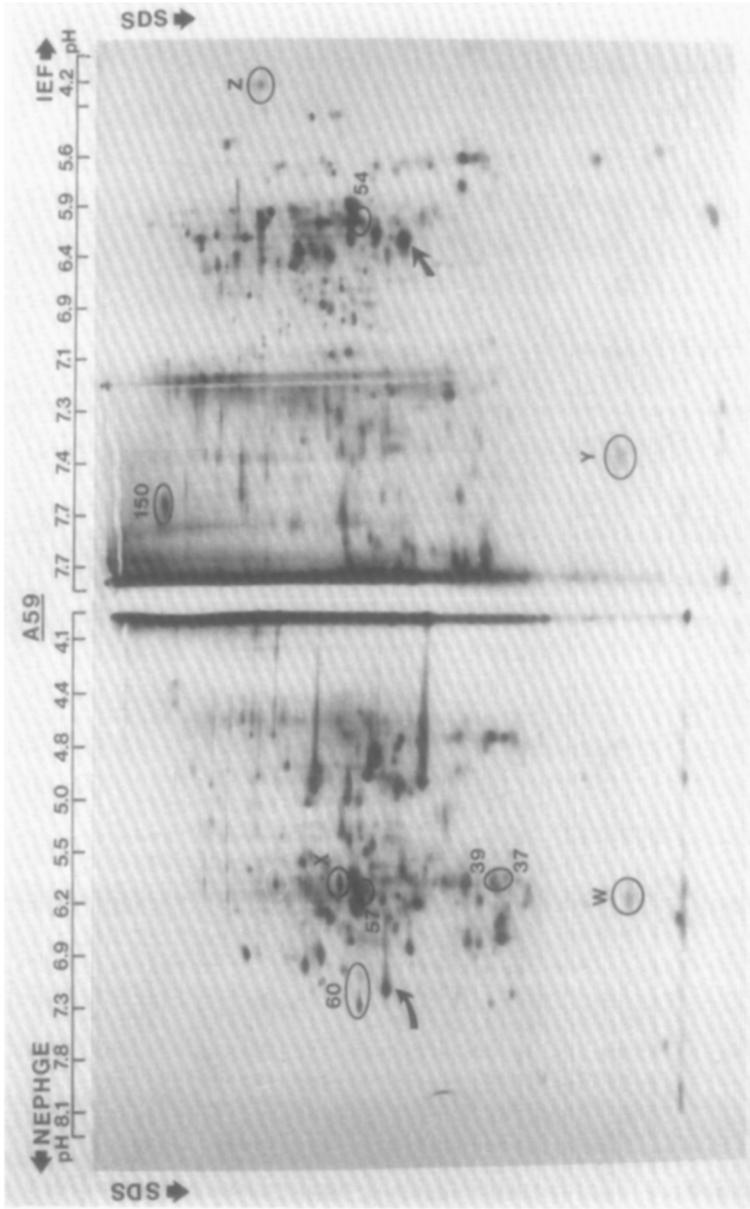


FIG. 8. Two-dimensional electrophoresis of proteins of cells infected by A59V. Details of the experiment are described in the legend to Fig. 7.

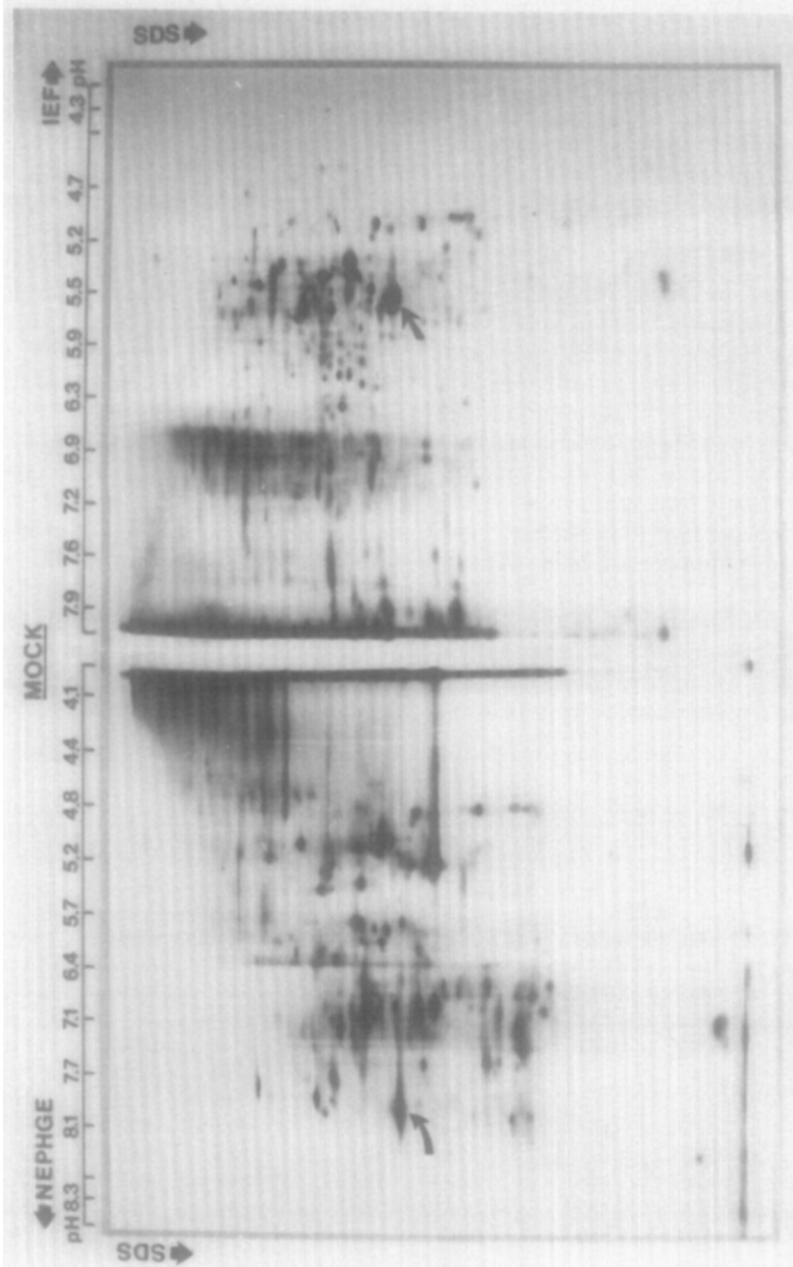


FIG. 9. Two-dimensional electrophoresis of proteins of mock-infected cells. Details of the experiment are described in the legend to Fig. 7.

first dimension, it is not possible to determine the *pI* of proteins, because NEPHGE is a nonequilibrium system (O'Farrell and O'Farrell, 1978). The very basic proteins 63,000 (JHMV) and 60,000 (A59V) comigrated with cytochrome *C* which has a *pI* of 9.8, suggesting that these proteins have a *pI* of 9.8 or greater. These proteins did not migrate as a single homogenous species. Instead, three or four spots can be seen suggesting that the proteins are heterogenous in charge, although homogeneous in size.

#### DISCUSSION

In this report we have identified nine virus-specific proteins in cells infected with either A59V or JHMV. Seven of these proteins were identified by one-dimensional SDS-polyacrylamide gel electrophoresis and their virus-specific nature was confirmed by radioimmune precipitation. We have also identified two additional virus-specific proteins by two-dimensional electrophoresis. A summary of this data is shown in Table 1.

Cells infected with either A59V or JHMV synthesize a virus-specific protein with a molecular weight of approximately 150,000. This protein is a structural protein present

on the surface of the virion (Sturman, 1977). This protein is a glycoprotein and is radiolabeled with mannose and glucosamine, but not fucose. Other investigators (Sturman, 1977; Sturman and Holmes, 1977) have found a glycoprotein of molecular weight 180,000 (E2) on the surface of the A59V virion which is radiolabeled by glucosamine and fucose. The discrepancy in the molecular weights obtained for this protein by ourselves and Sturman is probably due to the difficulty in sizing large glycoproteins. We have used the method of Segrest and Jackson (1972) in an attempt to correct for the anomalous migration of glycoproteins in SDS-polyacrylamide gels. The reason for the failure of this intracellular protein to radiolabel with fucose in our hands may be due to the addition of fucose to the protein during assembly of the virion.

We have identified a virus-specific protein with a molecular weight of approximately 90,000 in JHMV- and A59V-infected cells. We could not detect this protein by one-dimensional SDS-gel electrophoresis of cell lysates due to the high background of host-cell proteins. This protein was present in virions, was detected in cell lysates by two-dimensional electrophoresis,

TABLE 1

SUMMARY OF JHMV AND A59V VIRUS-SPECIFIC PROTEINS FOUND IN VIRIONS OR IN INFECTED CELLS

Virion <sup>a</sup>		RIP <sup>b</sup>		Cell lysates <sup>c</sup>				Function
One dimension		One dimension		One dimension		Two dimensions		
A59	JHM	A59	JHM	A59	JHM	A59	JHM	
150K	150K	150K	150K	150K	150K	150K	150K	Structural
89K	100K					89K	84K	Structural
						74K	70K	Nonstructural
60K	63K	60K	63K	60K	65K	60K	63K	Structural
		57K	61K	57K	61K	57K	61K	Nonstructural
		54K	56K	54K	56K	54K	56K	Nonstructural
		39K	39K	39K	39K	39K	39K	Nonstructural
		37K	36K	37K	36K	37K	36K	Nonstructural
20K	18K	20K	18K	20K	18K	20K	18K	Structural

<sup>a</sup> Virus-specific proteins in virions (See Fig. 5).

<sup>b</sup> Virus-specific proteins in radioimmune precipitates of infected cells (see Fig. 4).

<sup>c</sup> Virus-specific protein in whole cell lysates analyzed by either one-dimensional (see Figs. 2-4) or two-dimensional polyacrylamide gels (see Figs. 7-9).

and in some experiments was also detected by radioimmune precipitation. These results are in agreement with those of Sturman and Holmes (1977) who found a glycoprotein of this molecular weight to be present in A59 virions. The failure of this intracellular protein to radiolabel with sugars (Fig. 6) may be due to glycosylation occurring during virus assembly.

We have identified a virus-specific protein with a molecular weight of approximately 70,000 in A59V- and JHMV-infected cells. This protein was only detected by two-dimensional electrophoresis. This protein was present in multiple experiments, but its virus-specific nature should be considered tentative and needs confirmation.

We have identified an intracellular virus-specific protein with a molecular weight of approximately 20,000 (A59) or 18,000 (JHM). This protein is present in purified virus and probably corresponds to the glycoprotein of molecular weight 23,000 present in A59 virions described by Sturman (1977). The failure of this intracellular protein to label with sugars suggests that this protein may also be glycosylated during virion assembly. In addition, we occasionally see several proteins with molecular weights between 25,000 and 30,000 on two-dimensional gels of JHMV-infected cell lysates which are not present in mock-infected lysates or A59-infected cell lysates. We have not designated these as virus-specific proteins because of their inconsistent appearance. The presence of these proteins during JHMV infection may be dependent on the physiologic state of the cells at the time of infection or other presently undefined variables.

We have identified five other virus-specific proteins by one-dimensional and two-dimensional gel electrophoresis. The function of one of these proteins is known. The A59V-specific protein with a molecular weight of 60,000 and the corresponding JHMV-specific protein with a molecular weight of 63,000 are structural proteins present in the virion. These proteins are extremely basic and undoubtedly correspond to the arginine-rich core protein (VP50) described by Sturman (1977).

The noncoordinate synthesis of virus-specific proteins is not surprising. The presence of multiple mRNA species (Robb and Bond, 1979b) may be the basis of this noncoordinate translation. Further experiments should clarify the regulation of virus-specific transcription and translation.

#### ACKNOWLEDGMENTS

The excellent technical assistance of Carol Harris, Patricia Strack and Barbara Jean Miller is appreciated. This investigation was supported by Grants NS 12382 and NS 13898 awarded by the National Institute of Neurological and Communicative Disorders and Stroke, DHEW. J Robb is recipient of Research Career Development Award CA 70567, awarded by the National Cancer Institute, DHEW. C. Bond received support from Training Grant HL 07104, awarded by the National Heart and Lung Institute, DHEW and during part of this work was a Fellow of the Leukemia Society of America, Inc. J. Leibowitz is supported by Training Grant NS 07078, awarded by the National Institute of Neurological and Communicative Disorders and Stroke, DHEW.

#### REFERENCES

- BINGHAM, R. W. (1975). The polypeptide composition of avian infectious bronchitis virus. *Arch. Virol.* **49**, 207-216.
- BLATTLER, D. P., GARNER, F., VAN SLYKE, K., and BRADLEY, A. (1972). Quantitative electrophoresis in polyacrylamide gels. *J. Chromatogr.* **64**, 147-155.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- COLLINS, M. S., ALEXANDER, D. J., and HARKNESS, J. W. (1976). Heterogeneity of infectious bronchitis virus grown in eggs. *Arch. Virol.* **50**, 55-72.
- HIERHOLZER, J. C. (1976). Purification and biophysical properties of human coronavirus 229E. *Virology* **75**, 155-165.
- HIERHOLZER, J. C., PALMER, E. L., WHITFIELD, S. G., KAYE, H. S., and DOWDLE, W. R. (1972). Protein composition of coronavirus OC43. *Virology* **48**, 516-527.
- HIRANO, N., FUJIWARA, K., HINO, S., and MATUMOTO, M. (1974). Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch. Gesamte Virusforsch.* **44**, 298-302.
- LAEMMLI, U. K., and FAVRE, M. (1973). Maturation of the head of bacteriophage T4: I, DNA packaging events. *J. Mol. Biol.* **80**, 575-599.

- LASKEY, R. A., and MILLS, D. A. (1975). Quantitative detection of  $^3\text{H}$  and  $^{14}\text{C}$  in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**, 335-341.
- MAC NAUGHTON, M. R., and MADGE, M. H. (1977). The Polypeptide composition of avian infectious bronchitis virus. *Arch. Virol.* **55**, 47-54.
- MAIZEL, J. V., JR. (1971). Polyacrylamide gel electrophoresis of viral proteins. In "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. V, pp. 179-246. Academic Press, New York.
- O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.
- O'FARRELL, P. Z., GOODMAN, H. M., and O'FARRELL, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142.
- O'FARRELL, P. H., and O'FARRELL, P. Z. (1977). Two-dimensional polyacrylamide gel electrophoretic fractionation. In "Methods in Cell Biology, XVI" (G. Stein et al., eds.), pp. 407-420. Academic Press, New York.
- PIERCE, J., and SUELTER, C. H. (1977). An evaluation of the Coomassie brilliant blue G-250 dye-binding method for quantitative protein determination. *Anal. Biochem.* **81**, 478-480.
- ROBB, J. A. (1977). Identification of simian virus 40 tumor and U antigens. *Biochemistry* **74**, 447-451.
- ROBB, J. A., and BOND, C. W. (1979a). Coronaviridae. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 14, Plenum, New York, in press.
- ROBB, J. A., and BOND, C. W. (1979b). Pathogenic murine coronaviruses: I, Characterization of biological behavior *in vitro* and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59V viruses. *Virology* **94**, 352-370.
- SEGREST, J. P., and JACKSON, R. L. (1972). Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In "Methods in Enzymology" (V. Ginsbury, ed.), Vol. 28, pp. 54-63. Academic Press, New York.
- STUDIER, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**, 237-248.
- STURMAN, L. S. (1977). Characterization of a coronavirus: I, Structural proteins: Effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* **77**, 637-649.
- STURMAN, L. S., and HOLMES, K. V. (1977). Characterization of a coronavirus: II, Glycoproteins of the viral envelope: Tryptic peptide analysis. *Virology* **77**, 650-660.
- STURMAN, L. S., and TAKEMOTO, K. (1972). Enhanced growth of a murine coronavirus in transformed mouse cells. *Infect. Immun.* **6**, 501-507.
- WAGNER, R. R. (1975). Reproduction of rhabdoviruses. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 4, pp. 1-93. Plenum, New York.