PROTEOLYTIC CLEAVAGE OF PEPLOMERIC GLYCOPROTEIN E2 OF MHV YIELDS TWO 90K SUBUNITS AND ACTIVATES CELL FUSION

Lawrence S. Sturman[1] and Kathryn V. Holmes[2]

[1]Center for Laboratories and Research
New York State Department of Health
Albany, New York 12201

[2]Department of Pathology, The Uniformed Services
University of the Health Sciences
Bethesda, Maryland 20814

INTRODUCTION

During the past decade our laboratories have been studying the structure and functions of coronavirus glycoproteins. Several years ago, based on our studies with mouse hepatitis virus (MHV)-A59, we proposed a schematic model of the molecular organization of the coronavirus particle (Sturman et al., 1980). This model appears to be valid for most other coronaviruses as well (Garwes, 1980; Siddell et al., 1982). Our current representation of the structure of MHV-A59 is shown in Fig. 1 (Sturman and Holmes, 1983).

The virion contains three structural proteins: N, E1, and E2. N is a phosphorylated nucleocapsid protein which forms the helical nucleocapsid in association with the RNA genome. E1 is a small, matrix-like, transmembrane glycoprotein which is O-glycosylated in MHV and deeply embedded in the lipid bilayer. The viral peplomers, which are responsible for attaching the virus to cell-surface receptors, are composed of a large glycoprotein E2. It is not known how many oligomers of this N-linked glycoprotein made up each peplomer.

In this report we shall discuss the structure of E2 and its relation to an important biologic function, cell fusion. Fusion of cells by MHV-A59 will be correlated with trypsin-induced cleavage of the 180K form of E2 to two different 90K products.

P. J. M. Rottier et al. (eds.), *Molecular Biology and Pathogenesis of Coronaviruses*

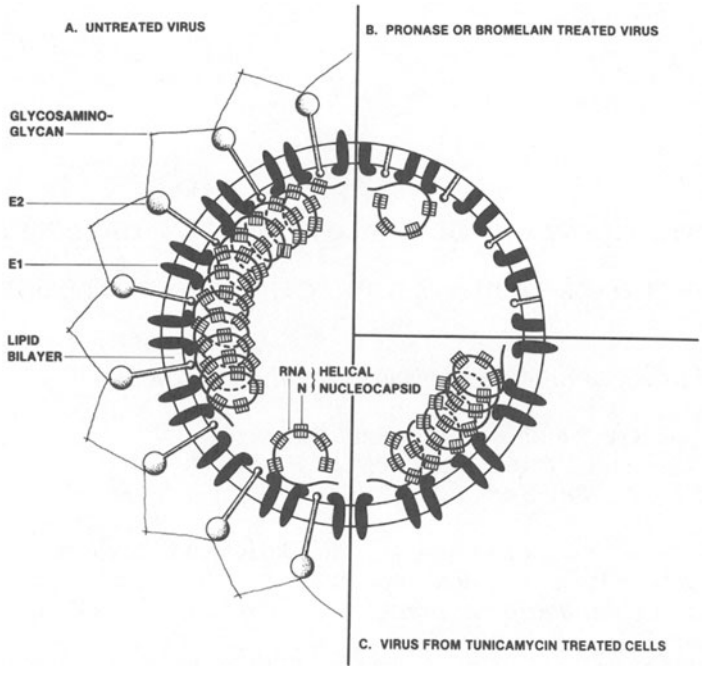

Fig. 1. Model for structure of MHV. (Copyright Academic Press; reprinted with permission from Sturman and Holmes, 1983)

EFFECTS OF TRYPSIN ON CORONAVIRUSES

The E2 peplomeric glycoprotein occurs in the virion in two forms with apparent molecular weights of 180,000 and 90,000 (Sturman and Holmes, 1977). We have shown previously that trypsin treatment of MHV quantitatively converts the 180K form of E2 to 90K forms and that the 180- and 90K components have identical tryptic peptide maps (Sturman and Holmes, 1977). At the time these observations were made, no functional role for proteolytic cleavage of E2 was known, and separation of the two 90K products was not achieved.

Trypsin influences in several ways the infection of cells by coronaviruses. Plaque formation of an enteropathogenic bovine coronavirus and several strains of infectious bronchitis virus (IBV) were enhanced by adding trypsin to the overlay (Storz et al., 1981; Otsuki and Tsubokura, 1981). In the presence of trypsin, infection with a bovine coronavirus was associated with cell fusion (Storz et al., 1981; Toth, 1982). Similar findings were obtained with some strains of MHV. Trypsin treatment of infected cells enable MHV-S to form fusion plaques on otherwise resistant cells and enabled MHV-2 to form fusion-type plaques (Yoshikura and Tejima, 1981).

The effects of trypsin on specific functions of coronavirus structural proteins have been more difficult to demonstrate. Initially trypsin treatment of virions appeared to be necessary for activation of IBV hemagglutinin (Corbo and Cunningham, 1959). Subsequently, however, the IBV hemagglutinin and the hemagglutinating activity of human coronavirus OC38/43 were shown to be inactivated by trypsin (Bingham et al., 1975; Kaye and Dowdle, 1969). Significant strain differences in IBV hemagglutination were noted, and the response of IBV to trypsin also differed according to the strain. Efforts to demonstrate an effect of trypsin on MHV infectivity gave equivocal results. Treatment of virions with low concentrations of trypsin enhanced infectivity two- to three-fold; treatment with high concentrations of trypsin reduced infectivity by a like amount (Sturman and Holmes, 1977).

ROLE OF E2 IN CELL FUSION

The role of E2 in induction of cell fusion was first indicated by the observations that (i) coronavirus-induced fusion was inhibited by monospecific anti-E2 antibody and (ii) treatment of infected cells with tunicamycin simultaneously inhibited the synthesis of E2 and cell fusion (Holmes et al., 1981). Monoclonal antibodies to E2 also inhibit cell fusion (Collins et al., 1982).

Direct evidence for the role of proteolytic cleavage of E2 in cell fusion has recently been obtained. Although other viruses, such as paramyxoviruses, can cause fusion after adsorption of concentrated virus but in the absence of virus replication, efforts to demonstrate rapid fusion by concentrated coronavirus were unsuccessful until we employed virus which had been pretreated with trypsin. No rapid fusion of L2 cells was produced by direct action of concentrated MHV-A59 (500 pfu/cell) on the plasma membrane (Fig. 2a). In this experiment no fusion was demonstrated until the end of the virus latent period (6 h). However, if the virions were pretreated with trypsin, which cleaved the 180K E2 to 90K forms, extensive cell fusion was observed by 75 min (Fig. 2b). This response was not affected by inhibition of protein synthesis with cyclohexamide. The pH optimum for cell fusion by concentrated virus was pH 7.2 to 7.8.

PURIFICATION OF 90A AND 90B CLEAVAGE PRODUCTS OF 180K E2

To clarify the relationship between the 180- and 90K forms of E2, we made use of the fact that the E2 glycoprotein is acylated (Niemann and Klenk, 1981). Purified virions labeled with [^{3}H] palmitic acid and ^{14}C-labeled amino acids were treated with trypsin, repurified by ultracentrifugation, and applied to hydroxyapatite columns in sodium dodecyl sulfate (SDS) after the method of Moss and Rosenblum (1972). Three major peaks of MHV-A59 proteins were eluted by a gradient of phosphate buffer at pH 6.4 (Fig. 3) and were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE; Fig. 4).

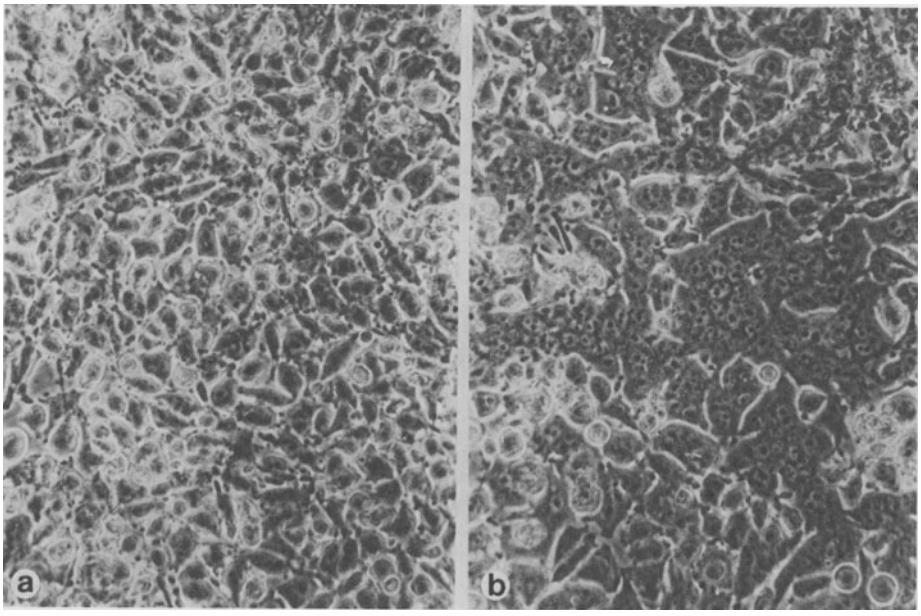

Fig. 2. Proteolytic activation of cell fusion by concentrated virus. (a) No fusion of L2 cells by direct action of concentrated MHV-A59 (500 pfu/cell) on the plasma membrane occurs within 2 h. (b) If the virions are pretreated with trypsin to cleave the 180K E2 to 90K forms, extensive fusion of cells is observed in 75 min.

The first peak contained almost all of the palmitic acid label and a 90K protein (Fig. 4A). However, this fatty acid was not covalently bound to the protein. The palmitic acid label from this peak migrated with the dye front in the gel and was probably associated with lipids in the viral envelope. The second peak contained the nucleocapsid protein N (Fig. 4B). The third peak consisted of two glycoproteins: the 23K matrix glycoprotein E1 and a 90K species which contained covalently bonded palmitic acid (Fig. 4C). Later fractions contained monomeric and aggregated forms of E1 (data not shown).

By analogy with other acylated viral glycoproteins, the fatty acid in E2 is probably located in the region at which the protein is anchored to the viral envelope. We have termed the 90K species which contained covalently bonded palmitic acid and which coeluted with E1, 90A. The other 90K species, which eluted first and did not contain palmitic acid label, we have termed 90B.

To demonstrate that the separation of 90A from 90B does not depend upon the interaction of 90A with E1 or the association of 90B with noncovalently bonded lipid, the two subunits of E2 were purified on hydroxyapatite following removal of the nucleocapsid, E1, and lipid. Trypsin-treated, purified virions were solubilized by NP40, the nucleocapsids were

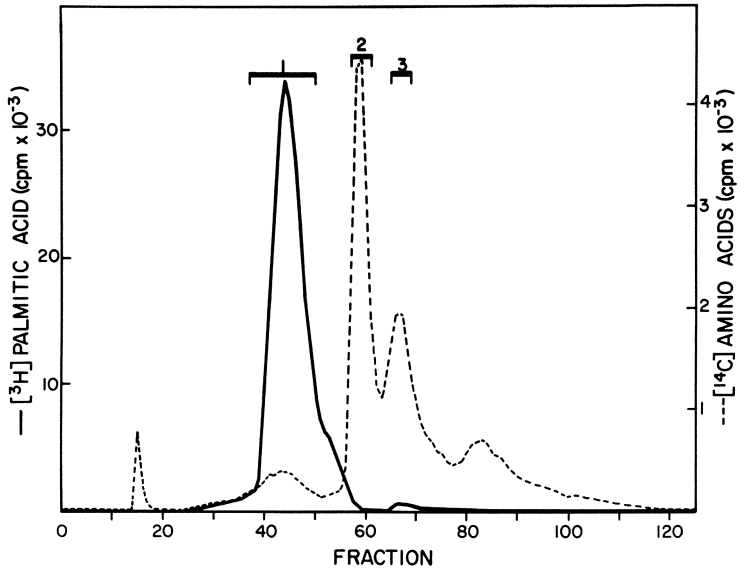

Fig. 3. Separation of structural proteins of MHV-A59 by chromatography on hydroxyapatite in SDS. Trypsin-treated MHV, labeled with ^{14}C-labeled amino acids and with [^{3}H] palmitic acid, was eluted in a gradient containing 0.2 to 0.5 M sodium phosphate, pH 6.4, with 0.2% SDS, 1 mM dithiothreitol.

removed by sucrose density gradient centrifugation (Sturman et al., 1980), and E2 was separated from E1 and lipid by Fractogel chromatography. The separation of NP40-solubilized E2 from E1 on Fractogel TSK (HW-55S) in 1% SDS is shown in Fig. 5. The first peak contained all of the fucose label but only a small fraction of the methionine. SDS-PAGE analysis revealed that this peak consisted of 90K E2, while the second peak contained E1. E2 was then separated into its 90A and 90B components by chromatography on hydroxyapatite or Ultrogel HA, a form of hydroxyapatite bound to agarose. The flow rates with Ultrogel were 5-10 times faster than with hydroxyapatite. Elution of [^{3}H] glucosamine and [^{35}S] methionine-labeled 90A and 90B from Ultrogel HA in 1% SDS with a gradient of phosphate buffer at pH 6.4 is shown in Fig. 6. Part of the 90B form eluted in the void volume.

AMINO ACID COMPOSITIONS OF 90A and 90B

Separation of 90A from 90B permitted comparison of their amino acid compositions (Table 1). The peak fractions containing 90A and 90B were concentrated by ultrafiltration with Millipore CX-30 filter units, and the residual SDS was removed by ion-pair extraction according to the method of Henderson et al. (1979). The amino acid compositions of 90A and 90B were determined, in collaboration with Dr. Thomas Plummer of

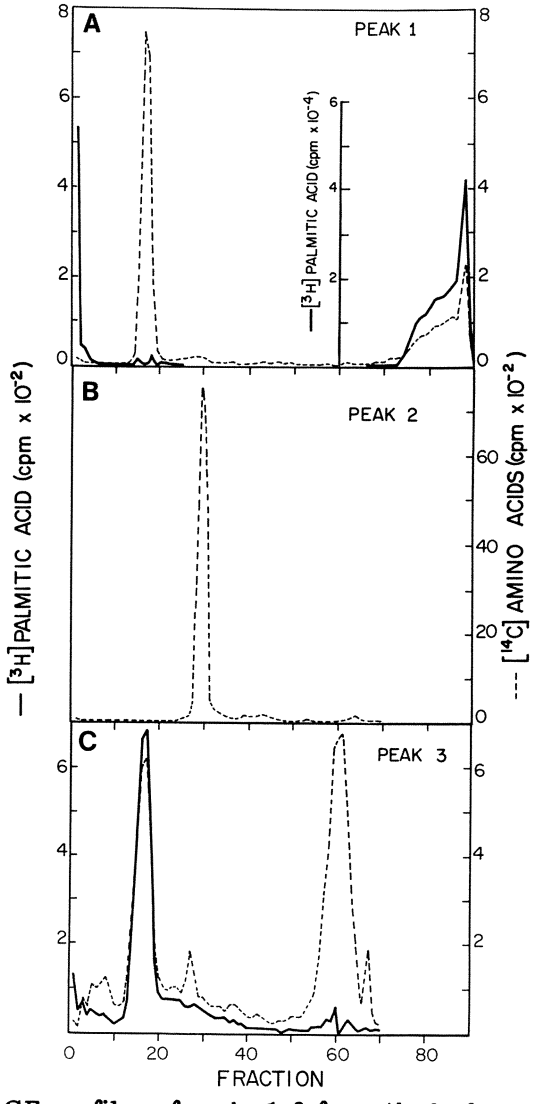

Fig. 4. SDS-PAGE profiles of peaks 1-3 from the hydroxyapatite column shown in Fig. 3.

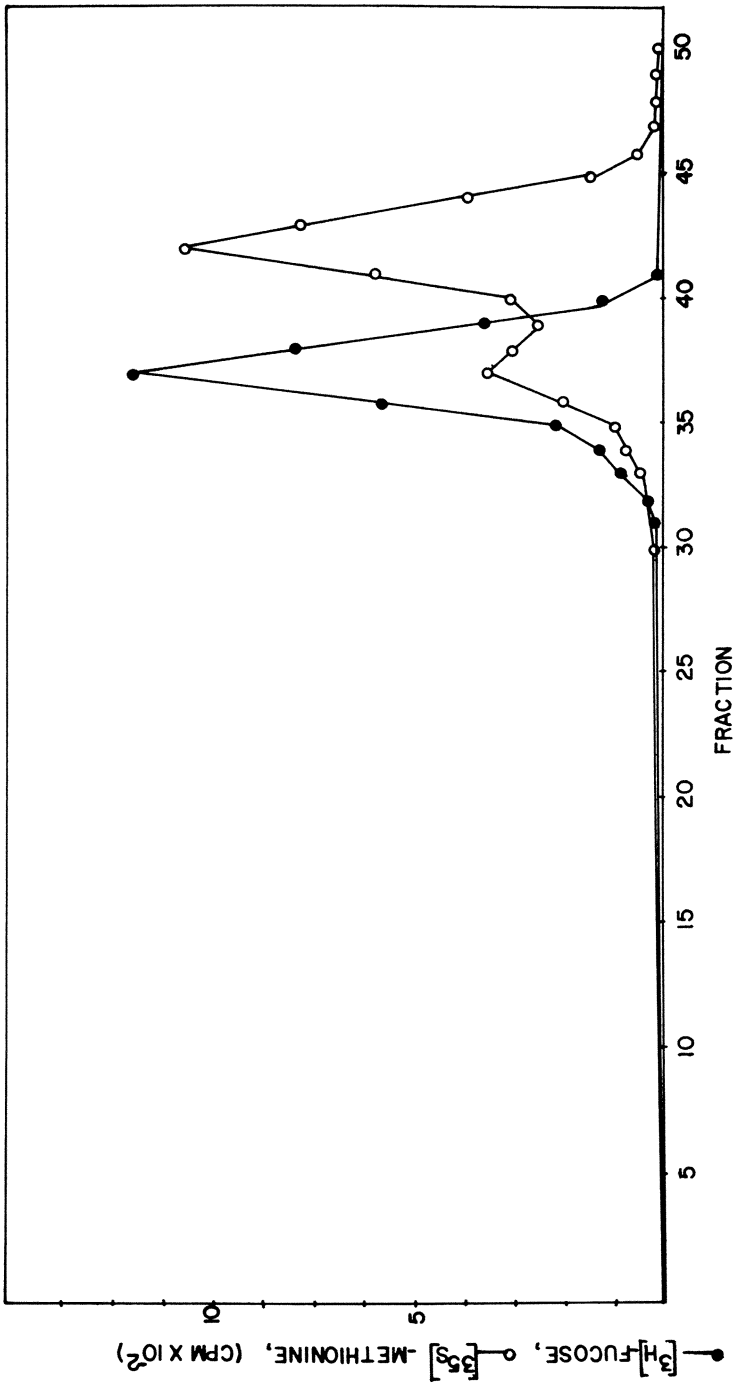

Fig. 5. Separation of E2 (90K) from E1 on Fractogel TSK (HW-55S) in 1% SDS, 0.05 M NaCl, 0.025 M sodium phosphate, pH 6.5. Trypsin-treated MHV was labeled with [^{3}H] fucose and [^{35}S] methionine.

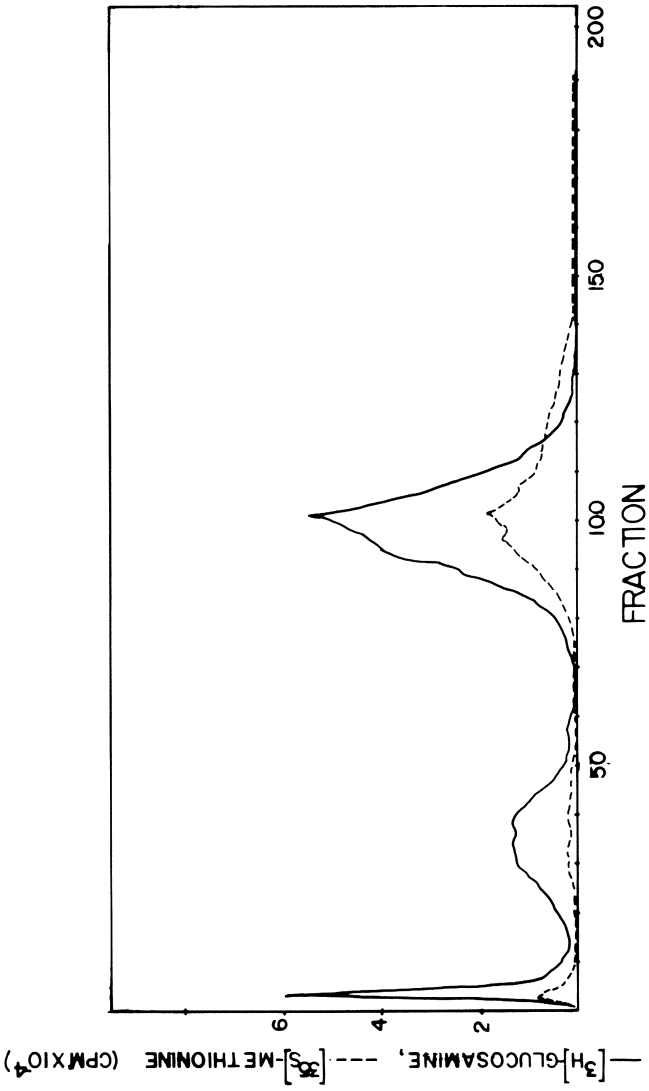

Fig. 6. Separation of 90A from 90B on Ultrogel HA in 0.15 to 0.45 M sodium phosphate, pH 6.4, 1% SDS.

this Center, on an amino acid analyzer after acid hydrolysis for 24 h.

As shown by the ratios of molar percentages (Table 1), threonine, serine, and glycine occurred in greater amounts in 90B, whereas the hydrophobic amino acids, valine, methionine, isoleucine, and leucine, were more abundant in 90A. These data support the conclusion that proteolytic cleavage of a large 180K form of E2 produces two different 90K products.

Table 1. Amino Acid Compositions of 90A and 90B Subunits of E2

	Mole Percent[b]		
Amino Acid[a]	90A	90B	90B/90A
Asx	11.06	11.51	1.04
Thr	6.63	7.97	1.20
Ser	6.67	8.40	1.26
Glx	11.30	11.25	1.00
Pro	4.29	4.06	0.95
Gly	8.96	11.46	1.28
Ala	7.81	6.78	0.87
Val	9.93	7.93	0.80
Met	1.33	0.87	0.65
Ile	4.64	3.78	0.81
Leu	9.31	7.63	0.82
Tyr	2.99	3.27	1.09
Phe	4.67	4.68	1.00
Lys	4.43	4.06	0.92
His	1.92	1.69	0.88
Arg	4.18	4.55	1.09

[a] Half-cysteine and tryptophan were not determined.
[b] Mean of three determinations.

CONCLUSIONS

The isolation of two different 90K cleavage products from the 180K E2 of MHV-A59 resolves the long-standing question of the relationship between the 90K and 180K forms of E2. Direct evidence for the role of proteolytic cleavage of E2 in activation of cell fusion suggests that cleavage of this large glycoprotein reveals a new active site, which has biologic functions in coronavirus infection and pathogenesis.

ACKNOWLEDGEMENTS

The authors thank C. Ricard, F. Baker, T. Plummer, C. Eastwood and M. Liebach, who participated in these studies. This work was supported by grants AI 18997 and GM 31698 from the National Institutes of Health and grant RO 7043 from the Uniformed Services University of the Health Sciences. The opinions expressed are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University School of Medicine of the Department of Defense.

REFERENCES

Bingham, R.W., Madge, M.H., and Tyrrell, D.A., 1975, Hemagglutination by avian infectious bronchitis — a coronavirus, J. Gen. Virol., 28:381.

Collins, A.R., Knobler, R.L., Powell, H., and Buchmeier, M.J., 1982, Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion, Virology, 119:358.

Corbo, L.J. and Cunningham, C.H., 1959, Hemagglutination by trypsin-modified infectious bronchitis virus, Am. J. Vet. Res., 20:876.

Garwes, D.J., 1980, Structure and physiochemical properties of coronaviruses, Colloq. Inst. Nat. Sante. Rech. Med., 90:141.

Henderson, L.E., Oroszlan, S., and Konigsberg, W., 1979, A micro-method for complete removal of dodecyl sulfate from proteins by ion-pair extraction, Anal. Biochem., 93:153.

Holmes, K.V., Doller, E.W., and Behnke, J.N., 1981, Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin, Adv. Exp. Med. Biol., 142:133.

Kaye, H.S. and Dowdle, W.R., 1969, Some characteristics of hemagglutination of certain strains of "IBV-like" virus, J. Infect. Dis., 119:576.

Moss, B. and Rosenblum, E.N., 1972, Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes, J. Biol. Chem., 247:5194.

Niemann, H. and Klenk, H.D., 1981, Coronavirus glycoprotein E1, a new type of viral glycoprotein, J. Mol. Biol., 153:993.

Otsuki, K. and Tsubokura, M., 1981, Plaque formation by avian infectious bronchitis virus in primary chick embryo fibroblast cells in the presence of trypsin, Arch. Virol., 70:315.

Siddell, S., Wege, H., and ter Meulen, V., 1982, The structure and replication of coronaviruses, Curr. Top. Microbiol. Immunol., 99:131.

Storz, J., Rott, R., and Kaluza, G., 1981, Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment, Infect. Immun., 31:1214.

Sturman, L.S., and Holmes, K.V., 1977, Characterization of a coronavirus. II. Glycoproteins of the viral envelope: Tryptic peptide analysis, Virology, 77:650.

Sturman, L.S., Holmes, K.V., and Behnke, J., 1980, Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid, J. Virol., 33:449.

Sturman, L.S., and Holmes, K.V., 1983, The molecular biology of coronaviruses, Adv. Virus Res., 28:35.

Toth, T.E., 1982, Trypsin-enhanced replication of neonatal calf diarrhea coronavirus in bovine embryonic lung cells, Am. J. Vet. Res., 43:967.

Yoshikura, H., and Tejima, S., 1981, Role of protease in mouse hepatitis virus-induced cell fusion, Virology, 113:503.