Structural Polypeptides of the Murine Coronavirus JHM

By H. WEGE, HANNA WEGE, K. NAGASHIMA AND V. TER MEULEN

Institute for Virology and Immunobiology, University of Würzburg,
Versbacher Strasse 7, 8700 Würzburg, W. Germany

SUMMARY

Analysis by SDS-polyacrylamide gel electrophoresis shows that the purified coronavirus JHM contains six polypeptides. The apparent mol. wt. of the polypeptides (GP1, GP2, GP3, VP4, GP5 and VP6) are 170000; 125000; 97500; 60800; 24800 and 22700, respectively. Four polypeptides are glycosylated (GP1, GP2, GP3 and GP5). The analysis of particles obtained after limited proteolysis with pronase suggests that GP2 and GP3 are protruding from the lipid envelope and, together with GP1, form the spike layer. Protein VP6 and a part of GP5 are located within the lipid bilayer. Protein VP4 is susceptible to digestion at a concentration of pronase which changes the morphology of the virus particles making the interior of the virus accessible. Subviral particles produced after treatment with the detergent Nonidet P40 banded at a higher density than the virus and contained only VP4, GP5 and VP6.

INTRODUCTION

In many animal species, including man, coronaviruses are associated with a great variety of diseases (McIntosh, 1974; Tyrrell *et al.* 1975). In many cases the viruses of this group are only characterized morphologically and serologically. Due to the lack of suitable tissue culture systems for virus propagation few reports on virus structure and replication of coronaviruses are available. The published data on structural proteins of these viruses are, in some cases, inconsistent (Hierholzer *et al.* 1972; Bingham, 1975; Garwes & Pocock, 1975; Collins *et al.* 1976; Hierholzer, 1976; MacNaughton & Madge, 1977; Sturman, 1977). The number of polypeptides reported for coronaviruses varies from 16 proteins (Bingham, 1975) for avian infectious bronchitis virus (IBV) to 4 proteins for the mouse hepatitis virus strain A59 (Sturman, 1977). Many of these differences may be accounted for by the methods of virus purification and protein analysis as well as by the tissue culture systems used in these studies.

For the murine coronavirus strain JHM no data on the structural polypeptides and the replication of the virus are available. This virus has recently gained interest since it induces in mice and rats a variety of central nervous system disorders which are used as models to study virus induced demyelination (Nagashima *et al.* 1978). In this communication, our findings on the polypeptide composition of JHM virus are presented and compared with the structural polypeptide patterns so far reported for other coronaviruses.

METHODS

Cells and viruses. The original stock of JHM virus was kindly supplied by Dr L. P. Weiner, Johns Hopkins University, Baltimore, U.S.A. The virus was grown on Sac(−) cells, a permanent rhabdosarcoma line from mice obtained from Dr M. Mussgay, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany. This cell line showed no induction of endogenous C-type viruses after infection with JHM virus. The plaque purified virus was propagated on cells grown in Roux bottles infected with 0·05 p.f.u./cell (adsorption for 1 h at 37 °C). The highest virus titres (up to 5×10^7 p.f.u./ml) were obtained using Eagle's minimal essential medium containing double the amount of amino acids and 5% foetal calf serum. The virus was harvested 20 to 24 h after infection, when more than 75% of the cells had formed syncytia.

Radioactive labelling of the virus. Twelve hours after infection the culture medium was replaced by minimal essential medium with 5% dialysed foetal calf serum including either 10 μCi/ml ^{3}H-amino acid mixture or 10 μCi/ml D-6-^{3}H-glucosamine hydrochloride or 5 μCi/ml ^{35}S-methionine. All attempts to grow the virus with media containing reduced amounts of amino acids, glucose or serum during the labelling period resulted in a decrease in the amount of infectious virus obtained.

Virus purification. All steps were performed at 4 °C. The medium from infected cells was clarified by centrifugation. After addition of NaCl to give 0·5 M and centrifugation at 10000 *g* for 20 min the virus was precipitated by addition of 1/2 vol. of polyethyleneglycol 6000 (30%, w/v, dissolved in NTE buffer consisting of 0·01 M-tris HCl, pH 6·5, 0·1 M-NaCl and 0·001 M-EDTA). After stirring for 10 min the virus was pelleted in a Sorvall GSA rotor at 10000 *g* for 30 min. The virus pellet was resuspended in NTE buffer using a Dounce homogenizer and centrifuged at 1000 *g* for 10 min to remove aggregates. The virus suspension was centrifuged through a linear gradient consisting of 22 ml 5 to 20% (w/w) sucrose on to a cushion (4 ml) of 60% (w/w) sucrose in NTE buffer at 70000 *g* for 2 h in a Beckman SW 27 rotor. After centrifugation, the 60% sucrose interface which contained the virus was harvested, diluted to less than 20% (w/w) sucrose and loaded on a linear gradient made of 20 to 60% sucrose (w/w). The virus was centrifuged to equilibrium at 55000 *g* for 12 h. The virus band was collected, diluted with NTE and used directly for enzyme or detergent treatment or, alternatively, pelleted at 90000 *g* for 90 min and immediately used for gel electrophoresis.

Analysis of polypeptides. The discontinuous SDS-polyacrylamide system of Laemmli (1970) was used. The separating gel, a slab of $12 \times 12 \times 0·2$ cm, consisted of a linear gradient of 7·5 to 15% polyacrylamide, pH 8·9 and a 5% stacking gel of pH 6·8. Samples were solubilized in 0·06 M-tris HCl (pH 6·8) with 2% SDS (w/v) and 0·1% β-mercaptoethanol before heating for 5 min at 56 °C. After electrophoresis the gels were further processed by the fluorographic method of Bonner & Laskey (1974). For comparison, cylindrical tube gels consisting of 10% polyacrylamide in the same buffer system or a continuous phosphate buffered system (Hierholzer, 1976) were used. The radioactivity of tube gels was determined by scintillation counting after slicing.

Analysis of Nonidet P40, pronase and trypsin treated purified virus. Purified virus was diluted with NTE buffer (pH 6·5) to reduce the sucrose concentration below 15% (w/w). For Nonidet P40 treatment, Nonidet P40 was added to give a final concentration of 1% (v/v) and after 15 min at 20 °C the virus was cooled immediately to 4 °C. For pronase P treatment pronase P, pre-incubated for 1 h at 37 °C, was added to the diluted purified virus. The mixture was incubated for 30 min at 37 °C and cooled to 4 °C. For trypsin treatment, trypsin
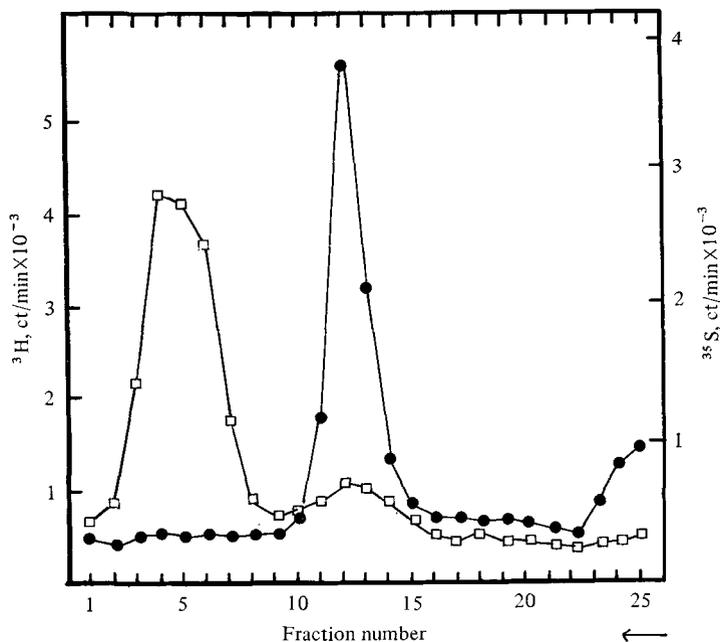
Fig. 1. Isopycnic density centrifugation of purified JHM virus before and after treatment with Nonidet P40. ●———●, JHM virus labelled with ^{35}S-methionine; □———□, JHM virus labelled with ^{3}H-amino acids and treated with 1 % Nonidet P40 for 15 min at 20 °C prior to centrifugation. Arrow indicates direction of sedimentation.

(1 mg/ml final concentration) was added to the diluted virus and the reaction was terminated after 1 h at 37 °C by addition of 3 mg/ml soya-bean trypsin inhibitor and cooling to 4 °C. In parallel to all treatments virus controls without enzyme or detergent were incubated under the same conditions.

The treated virus suspensions were loaded immediately on linear density gradients (20 to 60 % sucrose, w/w) and centrifuged to equilibrium as described above. The peak-fractions were pelleted and analysed by SDS-polyacrylamide gel electrophoresis.

Electron microscopy. Negative staining was done with 2 % potassium phosphotungstic acid at pH 6·0 using pure carbon coated grids. The specimens were investigated with a Zeiss-EM 10 microscope.

Materials. The following radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K.: ^{3}H-amino acid mixture (TRK 440), L-^{35}S-methionine (sp. act. 875 Ci/mmol), D-6-^{3}H-glucosamine hydrochloride (20 Ci/mmol). Acrylamide, N,N'-methylene bisacrylamide, sodium dodecylsulphate, pronase P (research grade), marker proteins (horse myoglobin, trypsin, chymotrypsin, pepsin, ovalbumin and bovine serum albumin) and soya-bean trypsin inhibitor were purchased from Serva. Trypsin (TPCK-treated, for biochemistry) came from Merck and Nonidet P40 from Shell.

RESULTS

SDS-polyacrylamide gel electrophoresis of purified virus

Purified virus had a buoyant density of 1·185 g/ml by equilibrium centrifugation (Fig. 1). Electron microscopic studies of these virus preparations revealed pleomorphic spherical particles with widely spaced spike projections (Fig. 6a).
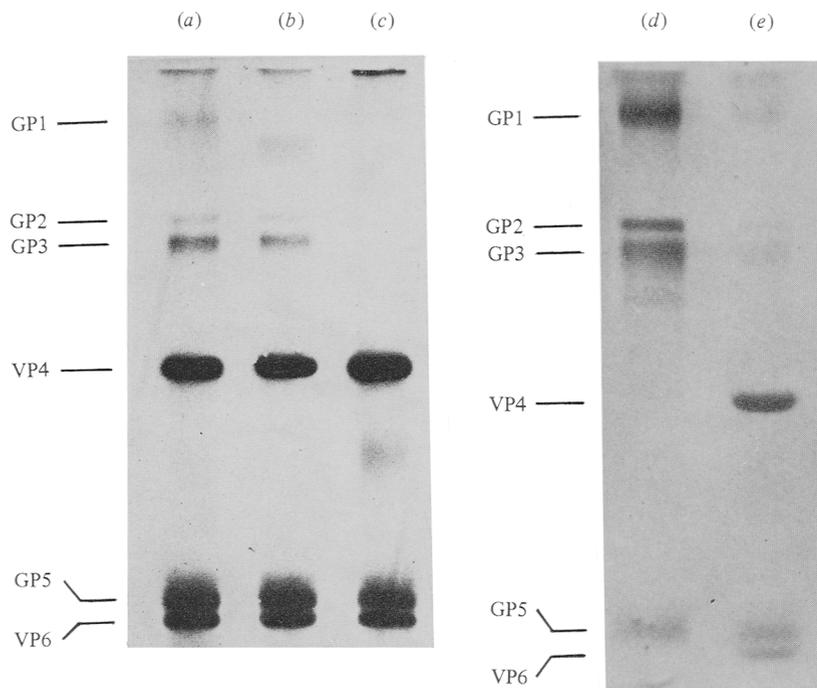
Fig. 2. Polyacrylamide gel electrophoresis of polypeptides of purified JHM virus labelled with (*a*, *b*, *c*, *e*) ³H-amino acids and (*d*) ³H-glucosamine. (*a*), untreated control; (*b*), treated with trypsin; (*c*), treated with Nonidet P40, subviral particle banding at high density; (*d*), co-migration of poly-peptides of virus labelled with ³H-glucosamine and (*e*), virus labelled with ³H-amino acids.

Pellets of purified virus grown in the presence of ³H-amino acids were lysed by treatment with 2% SDS and heating for 5 min to 56 °C. The polypeptide pattern obtained is shown in Fig. 2*a* and Fig. 3. Six polypeptides were found. The apparent mol. wt. were determined for 11 different virus batches by comparison with marker peptides run in parallel slots. The average mol. wt. values thus obtained are: 170000 ($\pm$ 10500); 125000 ($\pm$ 5000); 97500 ($\pm$ 3500); 60800 ($\pm$ 1500); 24800 ($\pm$ 1000); 22700 ($\pm$ 1000). The same number of polypep-tides and range of mol. wt. values was obtained by analysis with uniform cylindrical tube gels. Virus grown in the presence of ³H-glucosamine to identify glycopeptides revealed four glycosylated polypeptides (Fig. 2*d* and 4*a*). In Fig. 2(*d* and *e*) it can be seen by co-migration of purified virus labelled with ³H-glucosamine or ³H-amino acids that the polypeptide with a mol. wt. of 24800 and the three polypeptides of high mol. wt. are glycosylated. This particu-lar glucosamine labelled preparation revealed an additional band with a mol. wt. of approx. 85000 which was not repeatedly demonstrated. It cannot as yet be decided whether this component represents a structural polypeptide of the virus. This band was not found in virus preparations labelled with ³⁵S-methionine or ³H-amino acids. The following provisional nomenclature is used: GP1 (170000), GP2 (125000), GP3 (97500) and GP5 (24800) are virus glycopeptides, VP4 (60800) and VP6 (22700) are non-glycosylated polypeptides of the virion.

Treatment with trypsin

Digestion with trypsin (1 mg/ml, 60 min, 37 °C) only affected GP1 (Fig. 2*b*). This glyco-peptide migrated after treatment with an apparent mol. wt. of 150000. Analysis of virus
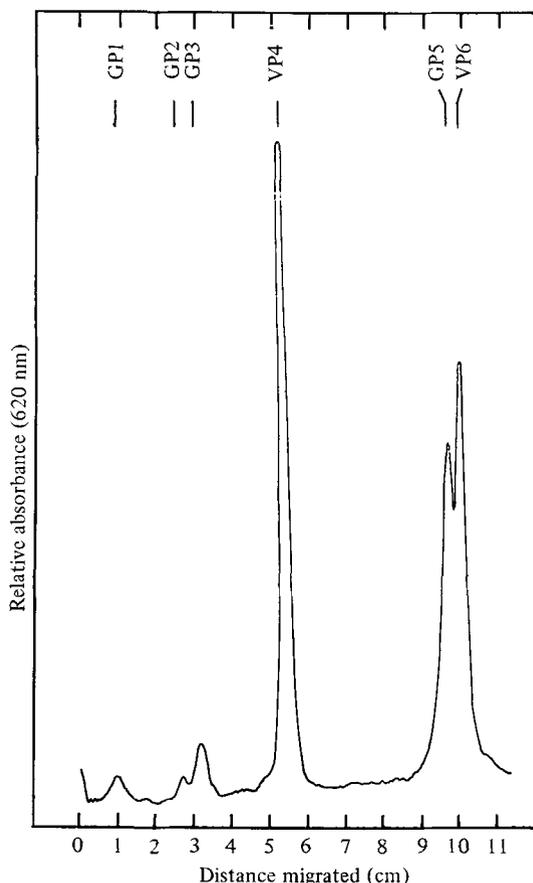
Fig. 3. Densitometer tracing of the polyacrylamide gel of purified JHM virus labelled with ³H-amino acids shown in Fig. 2(*a*).

proteins incubated with trypsin for periods greater than 1 h at 37 °C were inconclusive because the purified virus is heat labile and degradation products occurred in the enzyme-free control incubation.

Treatment with Nonidet P40

Purified virus labelled with ³H-amino acids was incubated for 15 min at 20 °C with 1 % (v/v) Nonidet P40 and re-centrifuged through a density gradient (Fig. 1). No virus banding at 1·185 g/ml was detected, but a broad peak corresponding to particles with a density of about 1·26 g/ml was obtained. These subviral particles were analysed by polyacrylamide gel electrophoresis (Fig. 2c). They contained only VP4, GP5 and VP6 and a broad, diffuse peak in the region corresponding to a mol. wt. of 40000. Electron microscopy of this material revealed rounded particles lacking the characteristic spike projections of complete virions.

Treatment with pronase

The effects of pronase on purified virus depended on the concentration and the duration of incubation. Virus preparations labelled with ³⁵S-methionine or ³H-glucosamine were re-purified after incubation with pronase (30 min, 37 °C) by centrifugation to equilibrium.
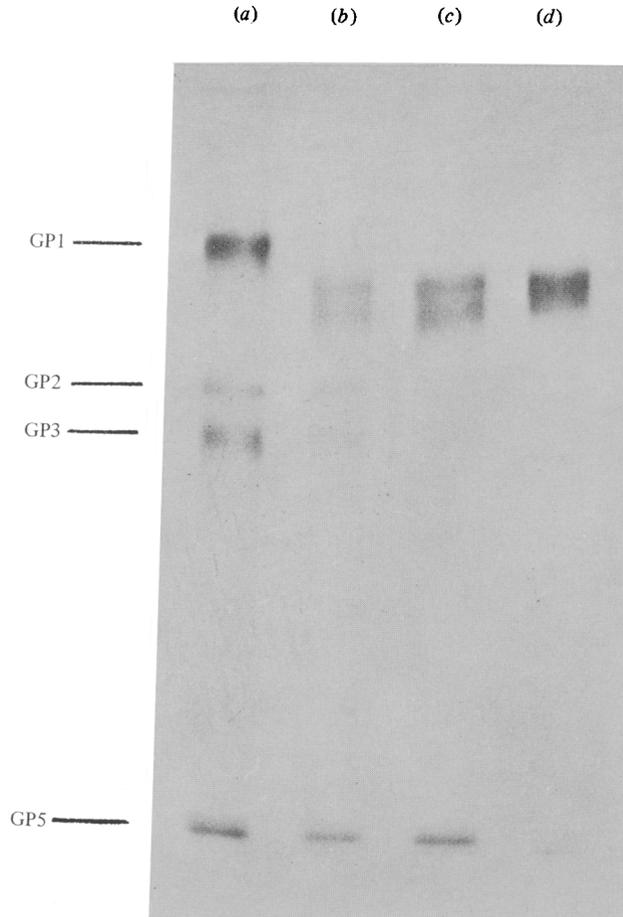
Fig. 4. Polyacrylamide gel electrophoresis of polypeptides of purified JHM virus labelled with
³H-glucosamine and digested with pronase (30 min, 37 °C). (a) Untreated control; (b) pronase
0·02 mg/ml; (c) pronase 0·1 mg/ml; (d) pronase 0·5 mg/ml.

Only a slight shift towards a lower density from an average of 1·185 to 1·175 g/ml was observed.

The results obtained under conditions of limited proteolysis using ³H-glucosamine labelled virus are shown in Fig. 4. The lowest pronase concentration applied (0·02 mg/ml) changed the apparent mol. wt. of GP1 to approx. 150 000. (Fig. 4b.) In this form the glycopeptide was also resistant to treatment with higher concentrations of pronase (Fig. 4c, d). After incubation with 0·1 mg/ml both GP2 and GP3 were degraded. GP5 was also affected by treatment with 0·5 mg/ml pronase (Fig. 4d). Virus labelled with ³⁵S-methionine (Fig. 5) showed exactly the same behaviour of GP1, GP2, GP3 and GP5 as described above. Polypeptide VP4 was only affected at a pronase concentration of 0·5 mg/ml. Polypeptide VP6 was minimally susceptible to proteolysis at any of the concentrations used. Data not shown indicated a slight decrease of the mol. wt. from 22800 to 19000. Virus labelled with ³⁵S-methionine showed two bands with a mol. wt. in the range of 40000 after pronase treatment, which were not visible with ³H-glucosamine-labelled virus.

Morphological changes obtained by treatment with 0·02 mg/ml and 0·1 mg/ml pronase
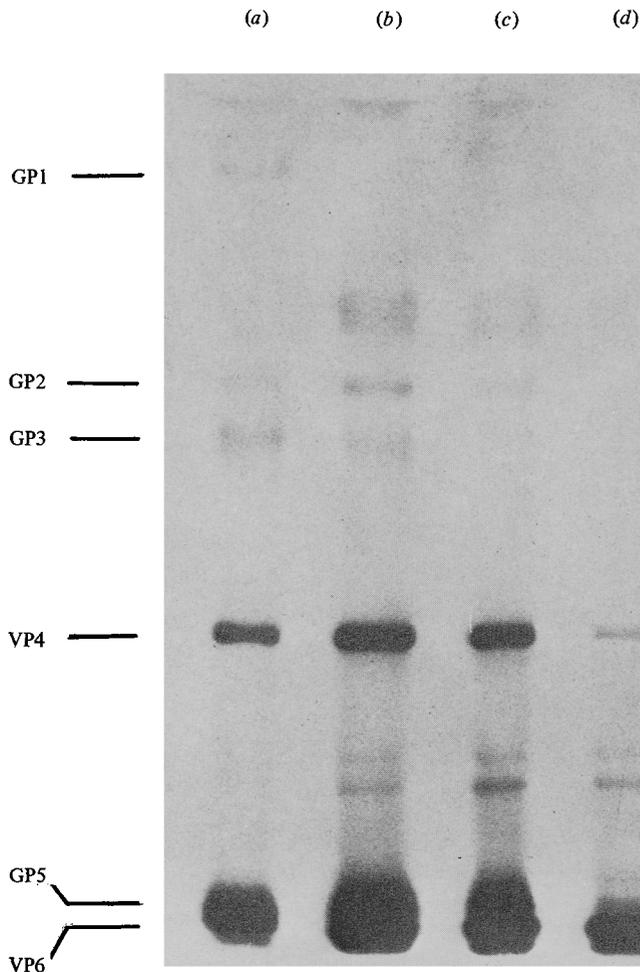
Fig. 5. Polyacrylamide gel electrophoresis of polypeptides of purified JHM virus labelled with ³⁵S-methionine and digested with pronase (30 min, 37 °C). (*a*) Untreated control; (*b*) pronase 0·02 mg/ml; (*c*) pronase 0·1 mg/ml; (*d*) pronase 0·5 mg/ml.

(30 min 37 °C) were investigated by electron microscopy. In contrast to the untreated control, nearly spikeless, rounded particles were observed which increased in number with the pronase concentration. After incubation with 0·5 mg/ml pronase the morphology of the re-purified particles was even more affected (Fig. 6*b*). At this stage the negative stain was penetrating the interior of the virus particles.

The effects of denaturing conditions on virus polypeptides

Harsher treatment of purified virus before electrophoresis than lysis in SDS and warming to 56 °C resulted in a more complicated pattern of the virus polypeptides. Similar effects have been described by Sturman (1977) for the murine coronavirus strain A59. The conditions routinely applied before gel electrophoresis involve boiling in the presence of urea and β-mercaptoethanol. Boiling of lysed JHM virus with 5 % β-mercaptoethanol for 2 min led to strong aggregation of proteins at the top of the gel, a decrease in GP1, GP5 and VP6 and a
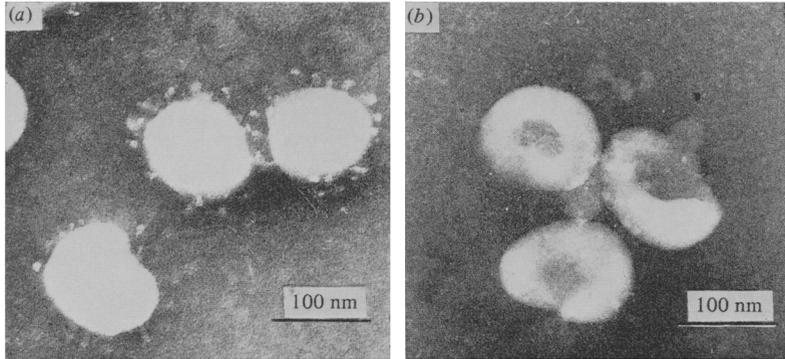
Fig. 6. Electron microscopy of purified JHM virus prepared by negative staining. (a) Untreated control; (b) pronase 0·5 mg/ml (30 min, 37 °C).
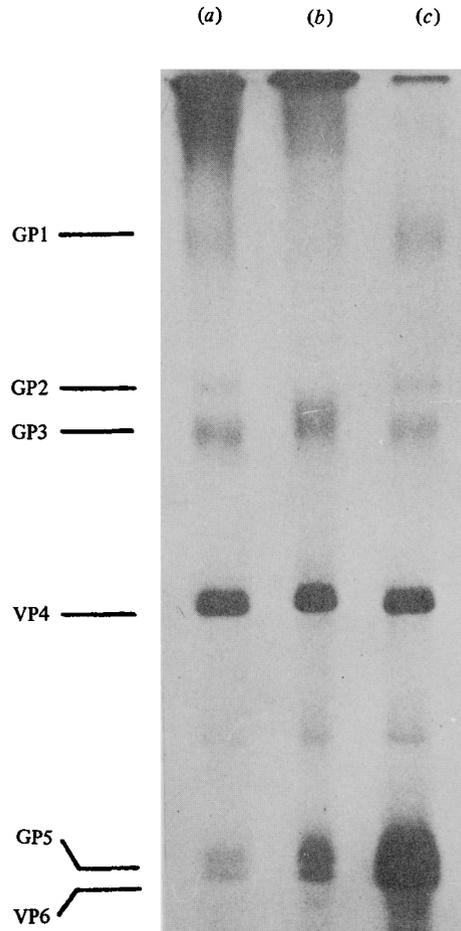
Fig. 7. Polyacrylamide gel electrophoresis of polypeptides of purified JHM virus labelled with ³⁵S-methionine and treated under different conditions after lysis with SDS: (a) 4 M-urea, boiled for 2 min; (b) 5 % β-mercaptoethanol, boiled for 2 min; (c) SDS only, boiled for 2 min.

disappearance of GP2 (Fig. 7*b*). It is also conceivable, that the effect of β-mercaptoethanol leads to a faster migration of GP2 resulting in a broad band together with GP3. Lysed virus, which was boiled in the presence of 4 M-urea, also showed heavy aggregates at the top of the gel and a decrease in GP5 and VP6 whereas GP2 remained unchanged (Fig. 7*a*). Boiling in the absence of urea or β-mercaptoethanol revealed an additional peptide with an apparent mol. wt. of 40000 which were not occurring by lysis at 56 °C and some aggregation on top of the gel (Fig. 7*c*). Similar effects were observed after prolonged storage of purified virus as a dry pellet at −70 °C or storage after lysis with SDS at −20 °C.

DISCUSSION

Analysis of purified JHM virus by SDS-polyacrylamide gel electrophoresis revealed six polypeptides, four of which were glycosylated. The findings differ from those obtained with another murine coronavirus, strain A59, which was shown to be composed of only four polypeptides, three of them being glycosylated (Sturman, 1977). Thus JHM virus contains one additional polypeptide in the high mol. wt. region and one additional polypeptide of low mol. wt. The number of polypeptides obtained for JHM was independent of the gel electrophoresis system applied, suggesting actual strain differences between the murine coronaviruses JHM and A59. No detailed data are available on the antigenic relationship of individual murine coronaviruses. The behaviour of JHM virus under conditions of limited proteolysis was different from A59 and it was not possible to correlate the patterns of proteolysis directly with the results reported for that strain. In particular limited proteolysis of A59 virus (Sturman, 1977) completely removed the two glycopeptides of high mol. wt. from the particles and resulted in a shift of GP23 to a highly resistant polypeptide with a mol. wt. of 18000. In contrast, treatment of JHM virus with pronase affected only a part of GP1, leaving the greater part of the molecule resistant against proteolysis. This would suggest that only a non-glycosylated fraction of GP1 is exposed to the action of pronase, the remaining part of GP1 being protected by glycosylation and attached to the lipid bilayer. Proteolysis with trypsin also seems to affect the non-glycosylated fraction of GP1 in the same manner. In strain A59 trypsin treatment was reported to split GP180 to GP90 (Sturman & Holmes, 1977). A similar dissociation of the high mol. wt. glycopeptide (GP1) to a molecule of approximately half its size (GP3) was not observed for JHM virus.

Further treatment with pronase removed GP2, GP3 and reduced markedly the amount of GP5. These digested particles did not reveal the characteristic spike layer when observed by electron microscopy, suggesting that the spikes are essentially composed of the glycopeptides. With ^{35}S-methionine labelled virus, two polypeptides with mol. wt. of 40000 and 35000 were seen after pronase treatment which were not obtained with ^{3}H-glucosamine labelled virus. These polypeptides might be non-glycosylated parts of the high mol. wt. glycopeptides located within the lipid bilayer and therefore not accessible to pronase.

Biochemical analyses of these preparations indicated that proteolysis with higher concentrations of pronase was affecting VP4 which is located inside the virion. Polypeptide VP6 remained relatively resistant against proteolysis and showed only a slight decrease of the mol. wt. to 19000, indicating that VP6 might be predominantly located within the lipid bilayer. Removal of lipids by the detergent Nonidet P40 produced a subviral particle which contained only VP4, GP5, VP6 suggesting that these polypeptides are connected to each other. Both the evidence that the glycopeptide GP5 is somehow linked to the internal polypeptide VP4 and that it is relatively resistant to pronase digestion supports the concept, that this protein is a transmembrane glycopeptide.

Subviral particles after treatment with Nonidet P40 were also demonstrated for two porcine (Garwes *et al.* 1976; Pocock & Garwes, 1977) and one human strain (Kennedy & Johnson-Lussenburg, 1976). The porcine subviral particles, analysed by polyacrylamide gel electrophoresis, revealed a polypeptide composition similar to JHM virus after treatment with Nonidet P40.

The available data on the structural proteins of coronaviruses from different species reveal some variety in number and size. In general, it appears that a coronavirus particle contains 4 to 7 proteins with two or more glycosylated polypeptides. All reports indicate the presence of a major non-glycosylated protein with a mol. wt. in the range of 50000 to 60000, located inside the virion. This probably represents the nucleocapsid protein. There are one or two polypeptides with mol. wt. below 50000, and at least one of these is glycosylated. A portion of these low mol. wt. molecules is located inside the lipid bilayer and connected to the internal polypeptide. The polypeptides of high mol. wt. (2 to 4) are all glycosylated and essentially involved in forming the spike layer.

The reports of two groups on the structure of avian bronchitis virus are inconsistent with the data obtained with other strains (Bingham, 1975; Collins *et al.* 1976). These authors found 14 to 16 polypeptides. In contrast MacNaughton & Madge (1977) and MacNaughton *et al.* (1977) investigating the same virus obtained only seven peptides, when avoiding highly reducing conditions and prolonged boiling before electrophoresis. It seems that coronavirus glycopeptides tend to form aggregates by boiling under highly denaturing conditions as it has been shown for the murine coronavirus strain A59 (Sturman, 1977) and JHM virus. It is conceivable that the high degree of glycosylation of some peptides hides hydrophobic parts within the polypeptides which are only revealed under high temperature and then give rise to aggregates upon cooling.

The functions of the different polypeptides of JHM virus are as yet unknown. It is also unknown in which manner some of the structural polypeptides of the virion are modified by post-translational processing. Further studies of the function and structure of the virus proteins and of the virus induced polypeptides in infected cells may lead to a model valid for all members of the coronavirus group.

REFERENCES

BINGHAM, R. W. (1975). The polypeptide composition of avian infectious bronchitis virus. *Archives of Virology* **49**, 207–216.

BONNER, W. M. & LASKEY, R. A. (1974) A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. *European Journal of Biochemistry* **46**, 83–88.

COLLINS, M. S., ALEXANDER, D. J. & HARKNESS, J. W. (1976). Heterogeneity of infectious bronchitis virus grown in eggs. *Archives of Virology* **50**, 55–72.

GARWES, D. J. & POCOCK, D. H. (1975). The polypeptide structure of transmissible gastroenteritis virus. *Journal of General Virology* **29**, 25–34.

GARWES, D. J., POCOCK, D. H. & PIKE, B. V. (1976). Isolation of subviral components from transmissible gastroenteritis virus. *Journal of General Virology* **32**, 283–294.

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. & DOWDLE, W. R. (1972). Protein composition of coronavirus OC43. *Virology* **48**, 516–527.

KENNEDY, D. A. & JOHNSON-LUSSENBURG, C. M. (1976). Isolation and morphology of the internal component of human coronavirus strain 229E. *Intervirology* **6**, 197–206.

LAEMMLI, U. I. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MACNAUGHTON, M. R. & MADGE, H. M. (1977). The polypeptide composition of avian infectious bronchitis virus particles. *Archives of Virology* **55**, 47–54.

MACNAUGHTON, M. R., MADGE, H. M., DAVIES, H. A. & DOURMASHKIN, R. R. (1977). Polypeptides of the surface projections and the ribonucleoprotein of avian infectious bronchitis virus. *Journal of Virology* **24**, 821–825.

MCINTOSH, K. (1974). Coronaviruses: a comparative review. *Current Topics in Microbiology and Immunology* **63**, 85–129.

NAGASHIMA, K., WEGE, H. & TER MEULEN, V. (1978). Early and late CNS-effects of coronavirus infection in rats. *Advances in Experimental Medicine and Biology* **100**, 395–409.

POCOCK, D. H. & GARWES, D. J. (1977). The polypeptides of haemagglutinating encephalomyelitis virus and isolated subvirual particles. *Journal of General Virology* **37**, 487–499.

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. & HOLMES, K. V. (1977). Characterization of a coronavirus II. Glycoproteins of the viral envelope: tryptic peptide analysis. *Virology* **77**, 650–660.

TYRRELL, D. A. J., ALMEIDA, J. D., CUNNINGHAM, C. H., DOWDLE, W. R., HOFSTAD, M. S., MCINTOSH, K., TAJIMA, M., ZAKSTELSKAYA, L. YA., EASTERDAY, B. C., KAPIKIAN, A. & BINGHAM, R. W. (1975). Coronaviridae. *Intervirology* **5**, 76–82.