STRUCTURAL CHARACTERIZATION OF IBV GLYCOPROTEINS

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INTRODUCTION

Avian infectious bronchitis virus (IBV) causes economically important disease in chickens, affecting both respiratory and non-respiratory tissues. Like other coronaviruses IBV virions contain three virus-coded protein structures, the spike (S; surface projection, peplomer), nucleocapsid (N) and matrix (M) proteins[1]. The polypeptides of M and N proteins have been identified. The M protein comprises a polypeptide of mol.wt. 23 000 (23K) which is glycosylated to different extents, the major glycopolypeptide being about 30K[2,3,4]. A polypeptide of about 50K forms the N protein[5]. However, although many papers have been published on the composition of IBV, there has been a distinct lack of agreement on the number and mol.wt. of the polypeptides which form the spikes (for references see[1,6].

Our initial work indicated that S comprised two glycopolypeptides S1 (90K) and S2 (84K)[7]. As will be shown, part of the confusion is because of the variable presence in virus preparations of contaminating host polypeptides which are sometimes present in quantities greater than the spike polypeptides. Also, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has shown that one of the S polypeptides (S2) stains poorly with Coomassie Brilliant Blue and has a tendency to aggregate.

Stern & Sefton[8] have recently shown, using tryptic peptide analysis, that S1 and S2 are derived from a common precursor molecule, showing that both S1 and S2 are virus-coded polypeptides. We show that purified S, from both radiolabelled and non-labelled virus, comprises both S1 and S2 and report on how these two glyco-

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polypeptides associate with each other to form S. In addition we have examined the nature of the oligosaccharides of S1, S2 and M.

METHODS

Radiolabelled and non-labelled IBV, strains M41 and D41, was prepared and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Cavanagh[4,6,7]. The preparation of IBV S protein has been described[6]. Briefly, IBV-Mr1 was dissociated with Nonidet P40 (NP40) and centrifuged at 85B g_{av} for 16h at 4°C in a 10-55% (w/w) sucrose gradient in NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM tris-HCl, pH 7.4) containing 0.1% NP40 and 1M NaCl or KCl until S had travelled about one third of the length of the tube. For the estimation of the mol. wt. of S the protein was used straight from preparative gradient. 25 ul containing S protein was diluted with 75/ul of NET buffer containing 0.1% NP40 and 5/ug of bovine catalase. This was layered on top of a 6 ml 5/20% (w/v) sucrose gradient in NET buffer containing 0.1% NP40 and centrifuged in a 3 x 6.5 ml MSE swing-out rotor at 4°C for 16h at 70K g_{av}. Fractions of 100/ul were collected and the constituent polypeptides detected by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, destained, and the S polypeptides visualised by silver staining[9]. Pre-staining with Coomassie Brilliant Blue enhanced the sensitivity of the silver stain. The mol. wt. of S was estimated using the formulae of Martin & Ames[10].

For studies with urea and SDS volumes of 100 or 200/ul of ^{35}S-methionine-labelled IBV-M41 in NET buffer containing sucrose (about 40% w/w) and 50/ug/ml of BSA were mixed with an equal volume of urea, in 5 mM tris-acetate pH 7.4 contining 50/ug/ml of BSA, or SDS, in NET buffer without BSA, at twice the desired final concentration. Control virus was mixed with buffer only. After 1h at 37°C (urea) or 30 min. at 25°C (SDS) the suspensions were diluted to 750/ul with NET buffer containing 50/ug/ml of BSA and loaded into 1 ml tubes and centrifuged in an MSE 3 x 6.5 ml swing-out rotor with the appropriate adaptor at 80K g for 2 h at 25°C. Pellets were recovered using 2% SDS and 2% 2-mercaptoethanol. For DTT treatment ^{35}S-methionine-labelled virus was incubated at 37°C for 1 h with an equal volume of buffer containing DTT at twice the desired final concentration. The virus was then sedimented to equilibrium in 25-55% (w/w) sucrose gradient at 30K g_{av} for 16 h at 4°C.

Endo- and exoglycosidases were used as described by Cavanagh[4] and in the legend to Fig. 1.

RESULTS

Glycosylation of IBV polypeptides

Stern et al.[2] have shown theat several polypeptides of IBV-Beaudette, analogous to polypeptides of 34K, 30K/28K, 26K and 23K

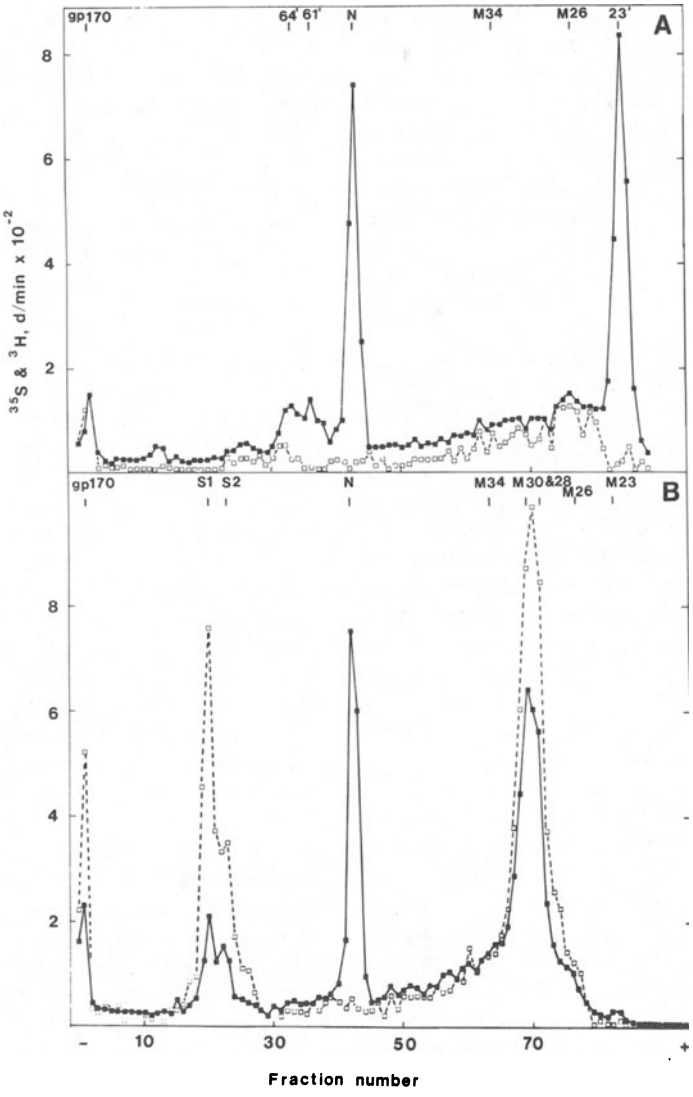

Fig. 1. SDS-PAGE of IBV-M41 labelled with ^{35}S-methionine (■) and ^{3}H-mannose (□) after incubation at 37°C for 40h at pH 6.0 with (A) a mixture of 25 mU/ml endoglycosidase H, 50 mU/ml endoglycosidase D, 50 mU/ml neuraminidase, 100 mU/ml b-N-acetylglucosaminidase and 100 mU/ml b-galactosidase, and (B) no enzyme. Both samples contained 1mM phenylmethylsulphonyl fluoride. 64', 61' and 23' refer to the mol. wt. (x10^{-3}) of polypeptides which are the products of hydrolysis by endoglycosidase H.

(M34 to M23) in our studies with IBV-M41, all give the same peptide maps. With the exception of M23, which is normally present in very small amounts, the M polypeptides of IBV were glycosylated, as shown by the incorporation of 3H-mannose (Fig. 1) and 3H-glucosamine. Analysis in 5 polyacrylamide gels of IBV-D41 labelled with 15 3H-labelled amino acids and ^{35}S-methionine showed that the 3H/^{35}S d/min (disintegrations per min) ratios for M23, M26, M28, M30 and M34 were 1.9, 1.9, 1.8, 1.9 and 1.9 respectively. Similar analysis of IBV-D41 labelled with 3H-glucosamine and ^{35}S-methionine gave 3H/^{35}S d/min ratios for M23, M26, M28, M30 and M34 of 1.2, 2.2, 3.2, 4.1 and 6.3 respectively. These data support the view of Stern et al.[2] that M23-M34 have the same polypeptide moiety but differ in their degree of glycosylation, this increasing with increasing mol.wt.

In order to determine whether the oligosaccharides of the S1, S2 and M polypeptides were N- or O-glycosidically linked, whether they were of the high mannose or complex type, and the proportion of the glycopolypeptide mol.wt. accounted for by the oligosaccharides, radio-labelled IBV-M41 was treated with endoglycosidase-H and -D. These enzymes remove high mannose and complex oligosaccharides respectively which are N-glycosidically linked to polypeptides.[11-13] The specificity of endoglycosidase H is such that if a polypeptide had only high-mannose oligosaccharides, endoglycosidase H would be expected to yield a polypeptide with some residual N-acetylglucosamine but no mannose. Such polypeptides were generated from S1, S2, M30 and M28. Endoglycosidase H removed oligosaccharides from both S1 and S2 of virus doubly labelled with ^{35}S-methionine and 3H-mannose (Fig. 1) or 3H-glucosamine. The products of hydrolysis were generally heterogenous and their mol.wt. varied among experiments. Mean mol.wt. values of 71K + 7K and 64K + 3K were obtained from 8 experiments. The smallest products, with mol.wt. of 64K and 61K, were obtained in the experiment shown in Fig. 1. Enzyme concentrations of up to 125 mU/ml did not decrease the size of S1 and S2 to less than in Fig. 1. Endoglycosidase D had no detectable effect on the S polypeptides by itself or with the exoglycosidases neuraminase, b-galactosidase and N-acetylglucosaminidase[13] without or with (Fig. 1.) endoglycosidase H. These results indicate that the oligosaccharides of S1 and S2 are N-glycosidically linked and are probably of the high-mannose type.

In all experiments the effect of endoglycosidase H on the M glycopolypeptides of IBV-M41 was the same: most of M30 and M28 were converted to a polypeptide of 23K with no mannose (Fig. 1.) and little glucosamine. Endoglycosidase D had no detectable effect on any of the M polypeptides. These results indicate that the oligosaccharides of M30 and M28 are N-glycosidically linked, high mannose side chains, and that the oligosaccharides of M34 and M26 are different from those of M30 and M28. This is in contrast to MHV and

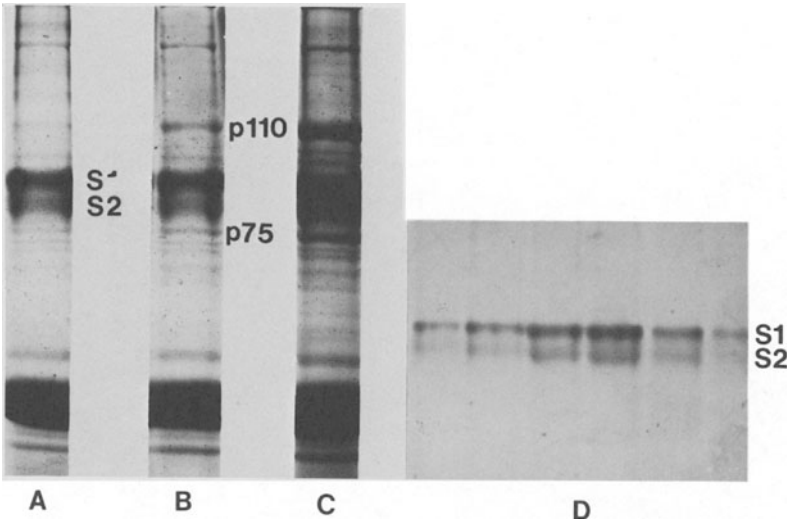

Fig. 2. SDS-PAGE of (a,b,c) 3 non-labelled preparations of IBV-M41; (d) non-labelled purified S polypeptides. IBV-M41 (2-4 mg of protein) was dissociated with 2% NP40 in 1M KCl in NET and sedimented in a 10-55% (w/w) sucrose gradient containing 0.1% NP40 and 1M KCl in NET. Only the S-containing fractions are shown in (d). Gels were stained with Coomassie Brilliant Blue. Only the half of the 10% acrylamide gel is shown.

bovine coronavirus L9 in which the matrix glycopolypeptide has exclusively O-linked oligosaccharides and lacks mannose[14]. The results above also indicate that the polypeptide moieties of S1 and S2 are about 60K, and that of M 23K.

Polypeptide of IBV Spikes

Analysis of non-labelled IBV-M41 polypeptides by SDS-PAGE and staining showed that all preparations contained S1 and S2, although S2 was less readily detectable than S1 unless the gel was heavily over-loaded with respect to N (Fig. 2A-C). In 75% of preparations S1 was the major polypeptide of > 54K, while in the remainder polypeptides of 110K (p110) and 75K (p75) were present in similar amounts to S1. In some preparations p110 and 75 were barely detectable. S prepared from non-labelled virus contained S1 and S2 but no other high mol.wt. polypeptides (Fig. 2D). The S of Fig. 2D was derived from a virus preparation which had a greater than usual amount of p110 and p75. However, these polypeptides remained near the top of the gradient, away from the S-containing fractions.

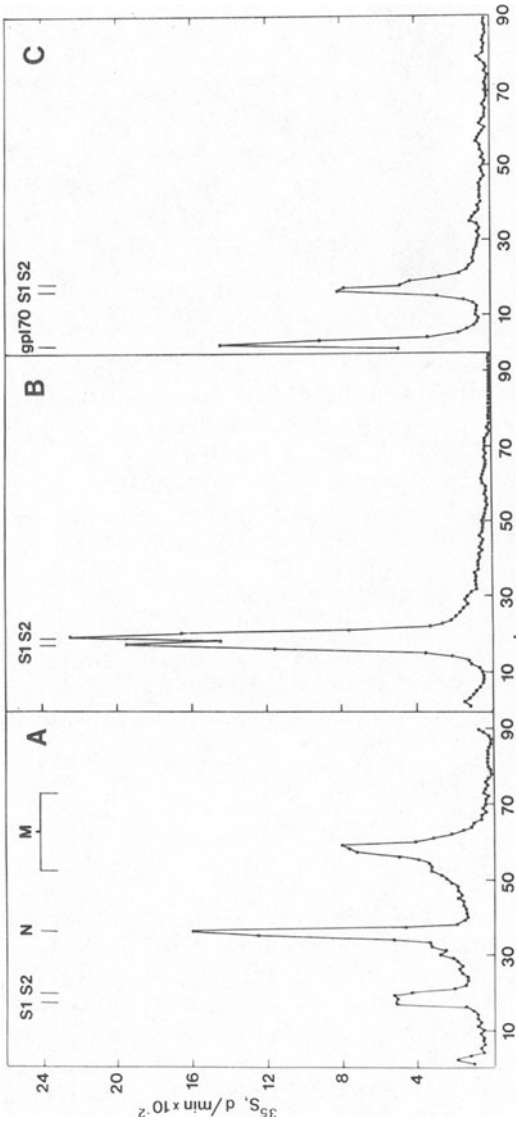

Fig. 3. SDS-PAGE of ^{35}S-methionine-labelled IBV-M41 polypeptides. Virus (a) was dissociated with 1% NP40 or 5% octyglucoside (OG) and sedimented in 10-55% (w/w) sucrose gradients containing 0.1% NP40 or 1% OG respectively. Three peaks of radio-label were obtained: (b) and (c) show the polypeptides present in the middle peak of the (b) NP40- and (c) OG-containing gradients respectively.

S1 and S2 were clearly present in the majority of radiolabelled IBV-M41 preparations (Fig. 6) and in the S isolated from them (Fig. 3). As can be seen in Fig. 3D, S2, and to a lesser extent S1, has a tendency to form aggregates with an apparent mol.wt. of 170K. We have observed that with IBV-Beaudette and other strains the aggregation of S2 is often greater than with IBV-M41. Dissociation of virus at 100°C did not consistently prevent this aggregation.

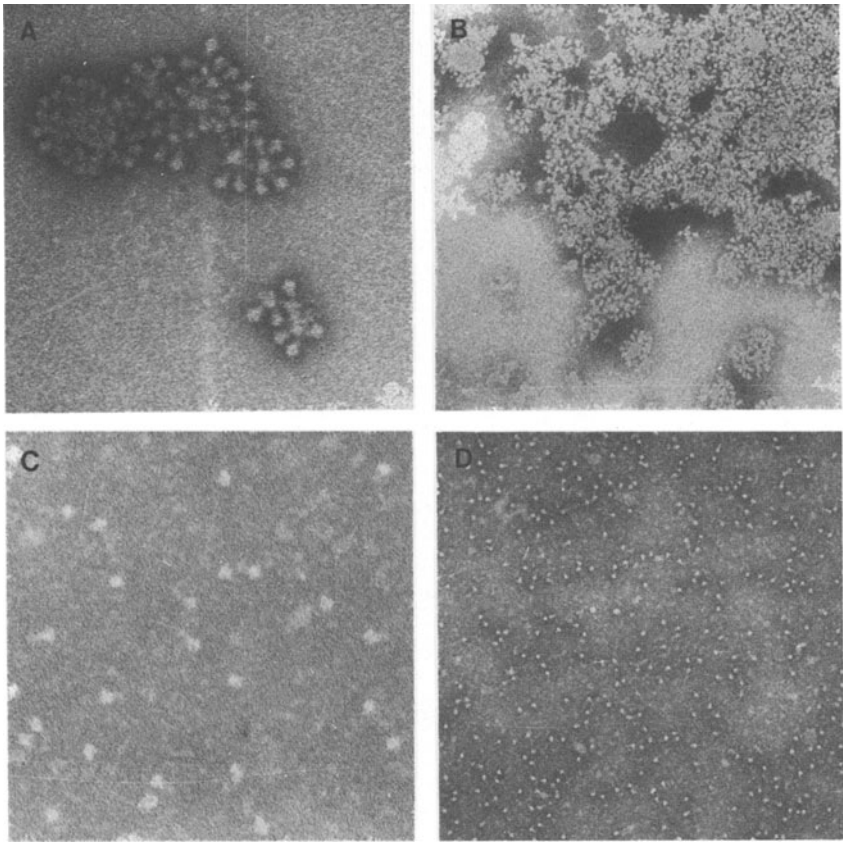

Fig. 4. Electron microscopy of purified spikes from IBV-M41. Spikes were separated from M and N protein by disruption of the virus with 2% NP40 and sedimentation in a 10-55% (w/w) sucrose gradient containing 0.1% NP40 and 1M NaCl. The spike containing fractions of one preparation were dialysed against water containing 0.1% NP40 lyophilized and resuspended to give a 10-fold concentration (a,b). Another preparation of spikes was examined without prior dialysis or lyophilization (c,d). Magnification (a,c) x 178000, (b,d) x 55000.

SDS-PAGE of IBV-M41 radiolabelled with a mixture of 15 3H-labelled amino acids and ^{35}S-methionine indicated that S contains S1 and S2 in a 1:1 molar ratio[6]. Radioimmunprecipitation with an anti-S monoclonal antibody precipitated equal amounts of S1 and S2 after dissociation of virus with NP40, a further indication that S1 and S2 are associated with each other (Mockett, A.P.A., Cavanagh, D. and Brown T.D.K. unpublished observation).

Molecular weight of S protein

The mol.wt. of S was estimated by co-sedimenting S with catalase in 5-20% (w/v) sucrose gradients containing NP40. Electron microscopy of non-labelled S which had been concentrated by dialysis and lyophilization showed that S had formed rosettes by aggregation at their narrow hydrophobic ends, and also larger aggregates by their bulbous hydrophilic ends (Fig. 4A,B). Such aggregates were obviously unsuitable for mol.wt. estimations. However, electron microscopy showed that S which had been neither dialysed nor concentrated had not aggregated (Fig. 4C, D) Consequently, S was used direct from preparative sucrose gradients. S sedimented in one band ahead of catalase (mol.wt. 250K) (Fig. 5) . A mol.wt. estimate for S of 354K ± 17K was obtained from three experiments. Ziemiecki & Garoff[15] have shown that the spikes of Semliki Forest virus, mol.wt.

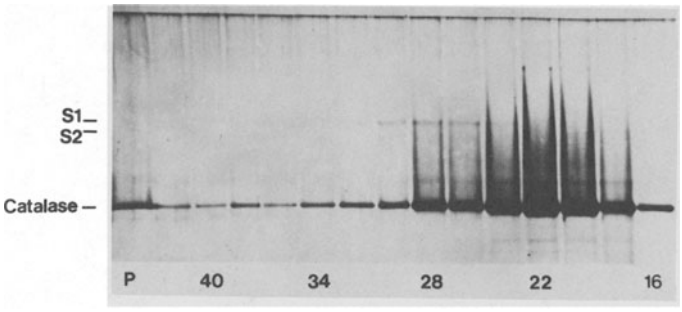

Fig. 5. Mol.wt. estimation of S from IBV-M41. Spikes were obtained as described for Fig. 5. and were not dialysed or lyophilized. S was co-sedimented with 5 µg of catalase in a 5-20% (w/v) sucrose gradient containing 0.1% NP40. Fractions of 100 µl were collected and 40 µl of alternate fractions analysed by SDS-PAGE followed by silver staining. Only the top part of the gel is shown. The right hand side of the gel corresponds to the upper part of the sucrose gradient. Pelleted material was resuspended in 140 µl of 2% SDS and 2% 2-mercaptoethanol and 40 µl (P) analysed in the gel.

111K, bound 0.21 mg of non-ionic detergent per 1.0 mg of protein. IBV S protein undoubtedly bound NP40 which would have led to an overestimate of the mol.wt. of S. The formulae used to calculate the mol.wt. of S apply most accurately to proteins which are essentially spherical molecules. The elongated shape of S would have reduced the sedimentation rate compared with a spheric molecule and would have resulted in an underestimate of the mol.wt.[16]. While these errors have not been quantified we know that the mol.wt. of S has to be multiples of 174K, since S comprises equimolar amounts of S1 and S2 [6]. Thus the mol.wt. estimate for S from sucrose gradient analysis of 354K ± 17K indicates strongly that S is a tetramer. Experiments with radiolabelled S which had been concentrated gave a major peak equivalent to that obtained with non-labelled S. In addition some S1 and S2 was present near the top of the gradient. In some preparations the amount of material in this peak was the same as in the 354K peak, indicating extensive breakdown of some spikes.

Nature of association of S1 and S2

After incubation of IBV-M41 at 37°C with 2M urea and pelleting of the virus, most of S1 had been removed from the virus (Fig. 6). Analysis of the supernatant by electrophoresis in tube gels showed that S1 was intact and was the predominant polypeptide present. The amount of S2, N and M present in the supernatants was very similar to that from the control virus. Essentially the same result was achieved with 6M urea.

Similar results were obtained with SDS (Fig. 7) as had been obtained with urea. The concentration of SDS that caused selective removal of S1 varied among experiments and was critical within each experiment. Thus in the experiment illustrated in Fig. 7. 0.01% SDS removed most of S1 (Fig. 7B), 0.015% removed even more (Fig. 7C) while 0.022% SDS disrupted more than 50% of the virus particles. Inspection of Fig. 7, confirmed by quantitative analysis of the polypeptides in the supernatants by tube gel SDS-PAGE, shows that 0.010% SDS caused the release of some N but not M polypeptide while even more N, but not M, was released by 0.015%.

These results indicate that S2 is more strongly associated with the virus membrane than S1. Indeed S1, may not be in contact with the membrane.

That S1 can be separated from S2 by urea or SDS alone indicates that disulphide bonds are not responsible for the association of S1 and S2. This was emphasized by the observations that IBV-M41 spikes were dissociated into their S1 and S2 components just as readily by SDS alone as by SDS with 2-mercaptoethanol at 100°C (Fig. 8) or 25°C. This also indicates that the two molecules of S1 per spike are not linked by disulphide bonds, and likewise for S2. After incubation of ^{35}S-methionine labelled IBV-M41 at 37°C for 1h with 100 mM

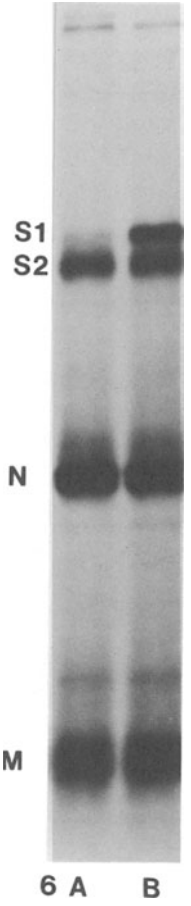

Fig. 6. Selective release of S1 by urea. ^{35}S-methionine-labelled IBV-M41 was incubated with or without (control) 2M urea for 1h at 37°C. The virus was pelleted and then analysed by SDS-PAGE in a slab gel and radiolabel detected by fluorography: (a) control, (b) urea-treated virus.

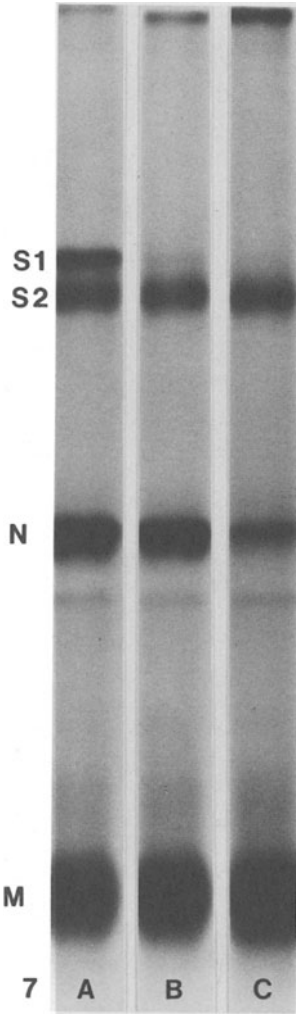

Fig. 7. Selective release of S1 by SDS. ^{35}S-methionine-labelled IBV-M41 was held at 25° for 30 min. with or without (control) SDS. The virus was pelleted and analysed as described in Fig. 6: (a) control; (b,c) virus after treatment with (b) 0.010% SDS and (c) 0.015% SDS.

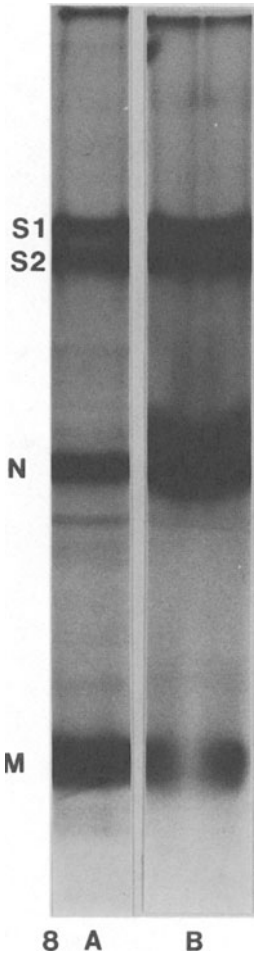

Fig. 8. ^{35}S-methionine-labelled IBV-M41 was heated at 100°C for 1 min with (a) 2% SDS and 2% 2-mercaptoethanol or (b) 2% SDS alone and then analysed as in Fig. 6.

dithiothreitol (DTT) the buoyant density of the virus in sucrose gradients was decreased from 1.18 g/ml to 1.16 g/ml. However, SDS-PAGE showed that no polypeptides had been removed. After incubation at 37°C for 1h of purified non-labelled spikes with 1 M DTT electron microscopy showed that the characteristic shape of the spikes had gone and also that they had aggregated much more than in the control. These results suggest that intrapeptide disulphide bonds do occur in S1 or S2 or both.

CONCLUSIONS

Results obtained with glycosidases have shown that S1, S2 and M30 and M28 of IBV have high mannose, N-linked oligosaccharides. The mol.wt. of the polypeptide moiety of the IBV M glycopolypeptide is very similar (23K) to that of murine hepatitis virus (MHV)[1] but the degree of glycosylation is much greater. This is probably related to the fact that the oligosaccharides of the M protein of MHV are of a different type and are O-linked to the M polypeptide[14].

Our results indicate that IBV S is an oligomeric protein comprising two molecules of each of the glycopolypeptides S1 and S2, to give a mol.wt. of approximately 350K. Under certain conditions[7] urea can remove both S1 and S2 from the virion, without disrupting the membrane or releasing M, indicating that S is a peripheral protein. Our more recent findings indicate that S2 anchors S to the membrane while S1 has little or no contact with the membrane. The association of S1 and S2 is not a strong one and intrapeptide, but not interpeptide, disulphide bonds exist in S. Fig. 9. is a simplistic representation of IBV S protein. This "ice cream cone" model is appropriate in that just as it is easy to lose ice cream from a cone while still holding the cone, so S1 is easily displaced from S2 while S2 remains in the membrane.

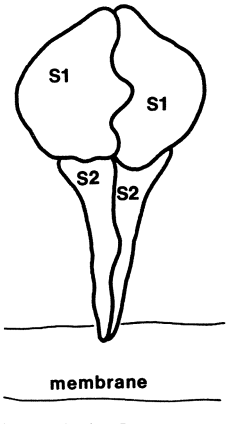

Fig. 9. A model for IBV S protein.

REFERENCES

1. S. G. Siddell, H. Wege and V. Ter Meulen. The structure and replication of coronaviruses. Curr. Top. Microbiol. Immunol. 99: 131 (1982).
2. D. F. Stern and B. M. Sefton. Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. J. Virol. 42: 208 (1982).
3. D. F. Stern and B. M. Sefton. Coronavirus proteins : structure and function of the oligosaccharides of the avian infectious bronchitis virus glycoproteins. J. Virol. 44: 804 (1982).
4. D. Cavanagh. Coronavirus IBV glycopolypeptides : size of their polypeptide moieties and nature of their oligosaccharides. J. Gen. Virol. 64: 1187 (1983).
5. M. R. Macnaughton, M. H. Madge, H. A. Davies and R. R. Dourmashkin. Polypeptides of the surface projections and the ribonucleoprotein of avian infectious bronchitis virus. J. Virol. 24: 821 (1977).
6. D. Cavanagh. Coronavirus IBV : further evidence that the surface projections are associated with two glycopolypeptides. J. Gen. Virol. 64: in press (1983).
7. D. Cavanagh. Structural polypeptides of Coronavirus IBV. J. Gen. Virol. 53: 93 (1981).
8. D. F. Stern and B. M. Sefton. Coronavirus proteins : biogenesis of avian infectious bronchitis virus virion proteins. J. Virol. 44: 794 (1982).
9. J. Morrissey. Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117: 307 (1981)
10. R. G. Martin and B. N. Ames. A method for determining the sedimentation behavior of enzymes : application to protein mixtures. J. Biol. Chem. 236: 1372 (1961).
11. A. L. Tarentino and F. Maley. Purification and properties of an endo-B-N-acetylglucosaminidase from Streptomyces griseus. J. Biol. Chem. 249: 811 (1974).
12. A. L. Tarentino, F. H. Plummer and F. Maley. The release of intact oligosaccharides from specific glycoproteins by endo-B-N-acetylglucosaminidase H. J. Biol. Chem. 249: 818 (1974).
13. N. Koide and T. Muramatsu. Endo-B-N-acetylglucosaminidase acting on carbohydrate moieties of glycoproteins. J. Biol. Chem. 249: 4897 (1974).
14. H. Niemann and H-D. Klenk. Coronavirus glycoprotein E1, a new type of viral glycoprotein. J. Mol. Biol. 153: 993 (1981).
15. A. Ziemiecki and H. Garoff. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. J. Mol. Biol. 122: 259 (1978).

16. R. Eason and A. M. Campbell. Analytical centrifugation, in "Centrifugal Separation in Molecular Biology and Cell Biology" G. D. Birnie and D. Rickwood, eds., Butterworths, London. (1978).