THE DISRUPTION OF INFECTIOUS BRONCHITIS VIRUS (IBV-41 STRAIN) WITH TRITON X-100 DETERGENT

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INTRODUCTION

The family *Coronaviridae* comprises a group of viruses with similar morphology which can cause disease in both mammals and fowl. All contain an outer lipid envelope from which radiate evenly-spaced projections 12—24 nm in lenght attached by a thin base to the membrane. Recent biochemical studies have shown that the genome of infectious bronchitis virus (IBV) is single-stranded RNA of approximate molecular weight of 5.4×10^6 and probably of positive polarity with respect to its capacity to act directly as a viral messenger RNA (Lomniczi, 1977; Lomniczi and Kennedy, 1977; Schochetman et al., 1977). Studies of virus structure have been extensively performed using several strains of IBV with widely varying results. In a previous paper from this laboratory, it was shown that the Massachusetts strain of IBV contained four major polypeptides species with apparent molecular weights of 90,000, 52,000, 29,000 and 26,000 (Lanser and Howard, 1980). Three of these structures with molecular weights of 90,000, 29,000 and 26,000 were found to be glycosylated as determined by the direct binding of radiolabelled concanavalin A to resolved components in polyacrylamide gels. The 29,000 molecular weight component appeared to be present as a dimer in the intact virus particle.

The use of detergents for the analysis of virus structure is well-established. When Hayman et al. (1973) solubilised influenza virus in sodium deoxycholate the released glycoproteins bound in the presence of the detergent to a column of *Lens culinaris* phytohaemagglutinin conjugated to Sepharose 4B. Extensive studies have been reported where the outer envelope glycoproteins of Semliki Forest virus have been successfully recovered without loss of antigenic quality by exposure to Triton X-100 (Helenius and Soderlund, 1973). Skelly et al. (1979) reported that hepatitis B surface antigen was effectively disrupted in the presence of 2% Triton and demonstrated that affinity chro-

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matography using columns of immobilised concanavalin A equilibrated in buffer containing detergent was effective in separating virus-specific glycoproteins from otherwise associated serum proteins.

In the present study, purified IBV was solubilised by exposure to Triton X-100 and the products separated either by affinity chromatography or by ultracentrifugation. The protein composition of each fraction was then determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in order to acquire some insight into the protein structure of this virus.

MATERIALS AND METHODS

Virus

The Massachusetts strain (M41) of infectious bronchitis virus was kindly supplied by Dr. Darbyshire of the Houghton Poultry Research Station, having previously been passaged 402 times in chickens and a further three times in embryonated hens' eggs. On receipt in the laboratory the virus was passaged a further 3 times in embryonated hens' eggs. Allantoic fluid was harvested 2 days after each infection and fluid from the third passage used as stock virus for all experiments. Aliquots stored frozen at -70°C until required.

Virus purification

The optimum conditions for the purification of virus from infected allantoic fluid have already been described (Lanser and Howard, 1980). Briefly, virus was precipitated from allantoic fluid by the addition of 8.7% w/v polyethylene glycol 6000 after the addition of NaCl to a final molarity of 0.46 M. The virus containing precipitate was collected after 12 h at 4°C and resuspended in 0.02 M Tris-HCl pH 7.0 containing 0.2 M glycine and 0.002 M EDTA (GNTE buffer). The concentrated virus suspension was then incorporated into the top 5 ml of a 5—45% w/v metrimazide (Nyegaard and Co., Oslo) gradient in GNTE buffer and centrifuged at 50,000 g in a Beckman SW40 rotor at 4°C for 8 h. The peak of virus infectivity was recovered and separated from metrizamide by G-75 Sephadex chromatography and the equilibrium centrifugation step repeated. This virus was again separated from metrizamide and stored at -70°C in GNTE buffer.

Disruption of virus with Triton X-100 detergent

Affinity chromatography
Purified virus was radiolabelled using the Bolton and Hunter reagent as previously described (Lanser and Howard, 1980) and solubilised by heating for 4 h at 37°C in 2% Triton X-100 (B.D.H. Chemicals Ltd., Poole) in GNTE buffer containing 0.5 M NaCl. Treated virus was then placed directly onto a 0.9 × 10 cm column packed with 6 ml

bed volume of Con A-Sepharose (Pharmacia Fine Chemicals) equilibrated in 0.1 M Tris-HCl buffer pH 7.3 containing 0.5 M NaCl, 2% Triton A-100, 1 mM CaCl$_2$. The eluant from the column was collected in 0.5 ml fractions and the level of radioactivity determined. After 30 ml had been collected from the column the eluant buffer was changed to 0.1 M Tris-HCl pH 7.3 containing 0.5 M NaCl, 2% Triton X-100 and 5% methyl mannoside. Fractions were again collected.

Fractions containing the radioactive peaks, i.e. those of the void volume and those which were eluted with methyl mannoside, were combined and dialysed overnight against GNTE buffer. The following day both peaks were treated with gelatinised Biobeads (Bio-Rad, Richmond, CA) to remove the detergent and analysed by SDS-PAGE.

Density gradient centrifugation

Purified virus was solubilised in 2% Triton X-100 in GNTE buffer containing 0.5% NaCl for 4 h at 37°C. Treated virus was then placed directly onto a 20–65% w/w sucrose gradient in GNTE buffer and centrifuged for 24 h at 150,000 g in Sorval AH-650 rotor. Protein bands were dialysed against GNTE buffer for 6 h at 4°C prior to analysis by electron microscopy and SDS-PAGE.

SDS-PAGE

Polypeptide analysis of solubilised IBV was performed by electrophoretic separation in 10% polyacrylamide gels using a high pH/discontinuous buffer system (Laemmli, 1970). Gels were prepared in 5 mm diameter precision glass tubes to a final length of 10 cm and contained 0.375 M Tris-HCl buffer pH 8.9. Resolving gels were overlaid with 1 cm stacking gel containing 4% acrylamide and buffered with 0.06 M Tris-phosphate buffer pH 6.7.

Samples for analysis were solubilised by heating for 2 min at 100°C in buffer containing 0.06 M Tris-phosphate pH 6.7 and 2% SDS. When required, 2-mercaptoethanol was added to a final concentration of 5% w/w. Electrophoretic separation was performed at a constant current with a maximum of 2.0 mA/gel. Bromophenol blue was added to all samples as a tracking dye.

Separated components were visualised by staining with 0.25% Coomassie Brilliant Blue dissolved in fixative solution (methanol (64) : acetic acid (23) : H$_2$O (64)). After 2 h at room temperature, gels were destained in 5% methanol and 7.5% acetic acid. Stained gels were scanned in a Joyce-Loebl Chromoscan 200 at 620 nm and the presence of radioactivity determined by counting 1 mm slices in a LKB Ultragamma Model 1280 well-type scintillation counter.

The presence of glycoproteins in stained polyacrylamide gels was determined by incubating 1 mm gel slices with ^{125}I-concanavalin A essentially as described by Skelly et al. (1979).

RESULTS

Polypeptides of infectious bronchitis virus

A total of three major polypeptides were resolved by PAGE analysis of radiolabelled virus (Fig. 1b), with estimated molecular weights of 90,000, 52,000 and 29,000, respectively. An identical pattern of separated polypeptides was obtained in Coomassie Blue-stained gels using unlabelled virus. In addition, two minor components with molecular weights of 38,000 and 48,000 were consistently present and two other minor components with molecular weights of 70,000 and 60,000 occasionally resolved. In order to confirm the specific association of these components with purified virus, radiolabelled material was reacted with a 1 : 100 dilution of chicken antiserum prior to PAGE analysis. All the major polypeptides were present in immune complexes, together with copies of the minor components described above (Fig. 1a).
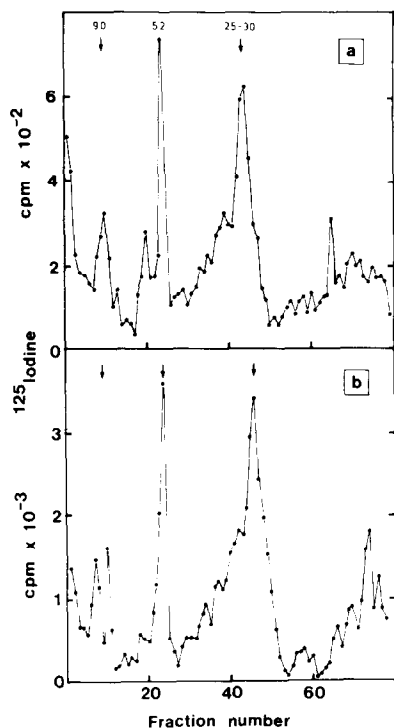

Fig. 1. SDS-PAGE of ^{125}I-IBV solubilised by heating at 100°C for 2 min in 2% SDS and 5% 2-mercaptoethanol and electrophoresed in 10% acrylamide gels. Major polypeptide components with estimated molecular weights of 90,000, 52,000 and 25 – 30,000 were present in samples analysed directly (panel b) or after reaction with 1 : 100 dilution of chicken antiserum (panel a).

Affinity chromatography on concanavalin A-Sepharose

Radiolabelled virus was solubilised in 2% Triton X-100 prior to chromatography. Fig. 2 shows the elution profile of radiolabel before and after the inclusion of 5% methyl mannoside in the column eluent. Approximately 67% of applied radiolabel remained unbound and was subsequently eluted in the void volume (fraction a). After elution of bound material with methyl mannoside, a further 2% of the radiolabel was recovered (fraction b). The remaining material was not recovered by increasing the concentration of methyl mannoside to 10%. Both fractions a and b contained radioactivity which was precipitated more than 95% in 10% trichloroacetic acid.

PAGE analysis of unbound and recovered material was performed after removal of the Triton X-100 detergent. Unbound radiolabel eluted early in fraction a was found to consist of the major 52,000 mol. wt. polypeptide species together with minor peaks or 42,000, 35,000 and 15,000 (Fig. 3a). The minor peaks did not correspond to any polypeptide found in immunoprecipitates of whole virus (Fig. 1). Suprisingly, no peaks were resolved in material eluted later in fraction a.

The bound material was recovered by the addition of methyl mannoside (fraction b) and was found to contain two polypeptides with molecular weights of 29,000 and 52,000, respectively (Fig. 3b). A polypeptide band corresponding to the 90,000 mol. wt. component present in whole virus was not resolved in either fraction. Since a proportion of the applied radioactivity was not eluted, the separation of solubilised components by ultracentrifugation was examined.
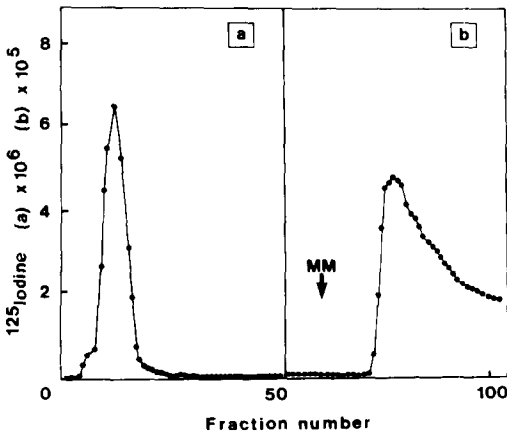

Fig. 2. Affinity chromatography of ^{125}I-IBV treated with Triton X-100. Radiolabelled virus was solubilised in 2% Triton X-100 in GNTE buffer containing 0.5 M NaCl for 4 h at 37°C. Disrupted virus was applied to a 0.9 × 10 cm column of concanavalin A-Sepharose 4B and washed with 0.1 M Tris-HCl buffer containing 0.5 M NaCl, 2% Triton X-100, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (panel a). Bound radioactivity was eluted by 5% α-methyl-D-mannoside in 0.1 M Tris-HCl (MM; panel b).
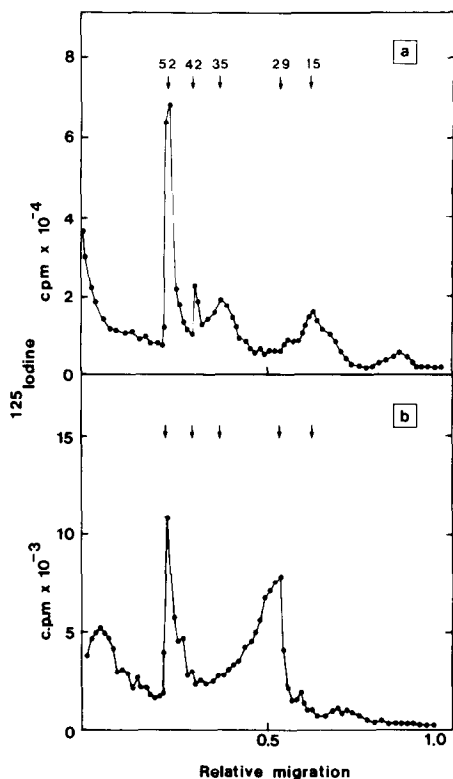
Fig. 3. SDS-PAGE analysis of ^{125}I-IBV fractionated by affinity chromatography. Aliquots from either the unbound fraction (Fig. 2a) or bound fraction (Fig. 2b) eluted from immobilized concanavalin A were solubilised and electrophoresed in 10% acrylamide gels as described in the legend to Fig. 1. Polypeptides were present with molecular weights of 52,000, 42,000, 35,000 and 15,000 in material from the unbound fraction (panel a) and polypeptides with molecular weights of 52,000 and 29,000 in the bound fraction (panel b). Arrows indicate position of polypeptides with their respective molecular weights indicated $\times$ 10^{-3}.

Equilibrium centrifugation in linear density gradients.

Two protein bands were resolved by the centrifugation of disrupted virus in linear sucrose gradients (Fig. 4). The lighter component possessed a buoyant density of 1.23 g/cm^3 and was found to contain three polypeptides with molecular weights of 90,000, 52,000 and 29,000, respectively (Fig. 5a). ^{125}I-concanavalin A labelling of the resolved components indicated that the 90,000 and 29,000 mol. wt. polypeptides were glycosylated (Fig. 6). Electron microscopy of this band showed the presence of small particles approximately 16 nm long which were similar to the spikes of the corona on intact virus.

PAGE of the second band with a buoyant density greater than 1.32 g/cm^3 resolved a non-glycosylated polypeptide of mol. wt. 52,000 (Fig. 5b) and a glycoprotein of mol. wt. 26,000 which could only be detected by reaction with ^{125}I-concanavalin A (Fig. 6b).
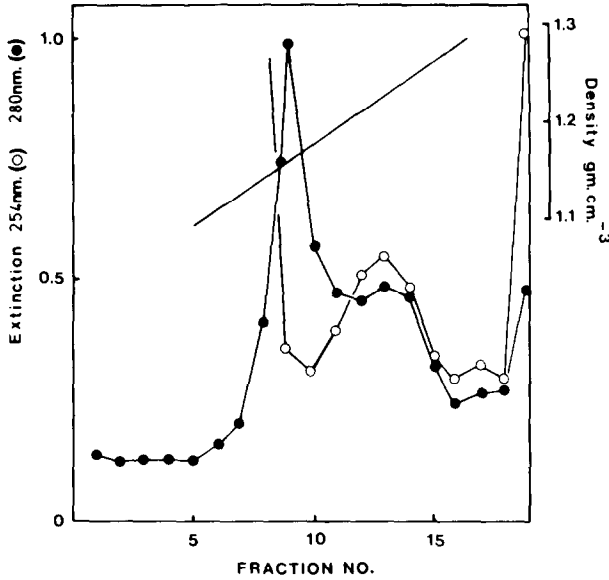
Fig. 4. Sedimentation of purified IBV solubilised with 2% Triton X-100 in a linear 20–65% w/v sucrose gradient. Centrifugation was for 24 h at 150,000 g. The gradient was fractionated into 0.25 ml aliquots and the extinction determined at 280 nm (●——●) and 254 nm (○——○) for each fraction.
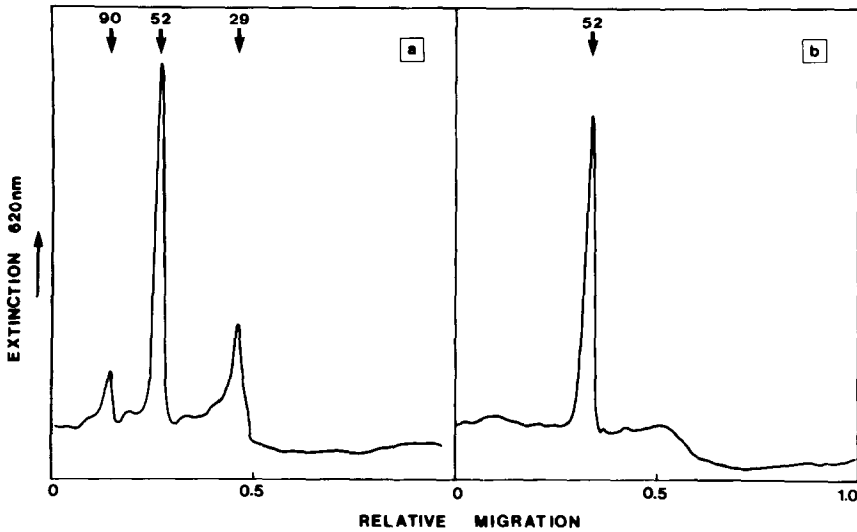

Fig. 5. SDS-PAGE analysis of IBV polypeptides separated by centrifugation in a sucrose gradient after solubilisation with 2% Triton X-100. Material recovered at a density of 1.23 g·cm⁻³ contained the three major virion polypeptides with molecular weights of 90,000, 52,000 and 29,000 (panel a). Protein recovered from the pellet contained only the 52,000 mol. wt. component (panel b). Molecular weights indicated X 10⁻³. Polypeptides were visualised by staining with Coomassie Brilliant Blue.
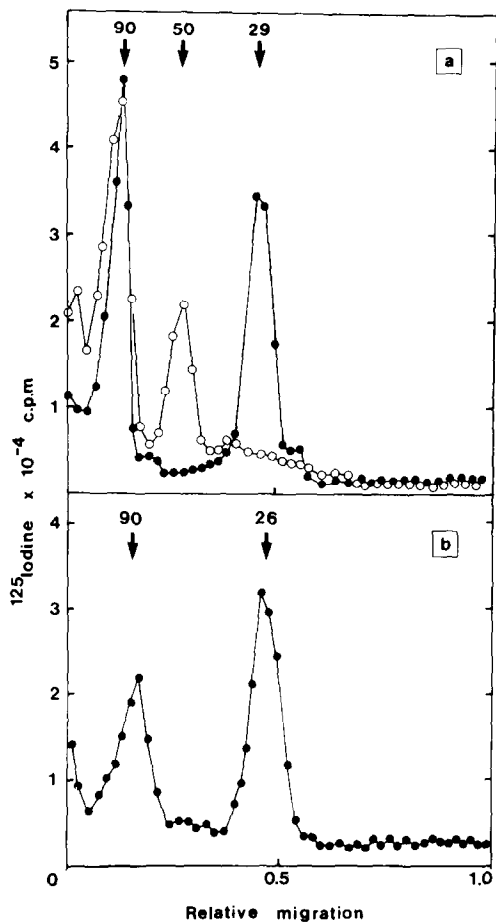
Fig. 6. Identification of glycosylated components present in stained acrylamide gels illustrated in Fig. 5. Viral protein recovered at a density of 1.23 g·cm⁻³ after solubilisation with 2% Triton X-100 contained two glycopeptides with molecular weights of 90,000 and 29,000, respectively (●—●, panel a). In the absence of 2-mercaptoethanol, the 29,000 mol. wt. component is identified as a glycosylated dimer of mol. wt. 50,000 (○—○). Material recovered in the pellet after sucrose gradient centrifugation contained two glycopeptides with molecular weights of 90,000 and 26,000, respectively (panel b).

Similar results were also obtained with the use of linear 10—50% metrizamide gradients (data not shown). The lighter band with a buoyant density of 1.22 g/cm³ contained particles similar in appearance to the particles banding at a buoyant density of 1.23 g/cm³ in sucrose. The denser subviral particles pelleted through a density of 1.32 g/cm³ in metrizamide and were visualised as aggregates of spheres 25—45 nm in diameter by electron microscopy. Similar experiments performed using radiolabelled virus resulted in the absence of radioactivity in the high density fractions, suggesting that the viral core components were not radiolabelled by the use of the Bolton and Hunter reagent.

DISCUSSION

Several comparative studies have investigated the structure and chemical composition of avian and mammalian coronaviruses. Although analyses of different coronaviruses have produced considerable variation in both the estimated size and number of viral gene products, there is general agreement that the major polypeptide associated with the 'core' component is a non-glycosylated polypeptide with a molecular weight in the range of 50–55,000 (Garwes and Pocock, 1975; Hierholzer, 1976; Sturman, 1977). In contrast, there has been considerable variation in the reported composition of the remaining structural components. In a recent report from this laboratory (Lanser and Howard, 1980) it was shown that the Massachusetts strain (IBV-41) of infectious bronchitis virus contained in addition at least three glycosylated polypeptide structures with molecular weights of 90,000, 29,000 and 26,000, respectively. In the absence of 2-mercaptoethanol, the 29,000 mol. wt. component migrated in acrylamide gels as a dimer in the 50,000 mol. wt. region. Each of these components is present in immune complexes of purified virus and chicken antiserum to this strain of IBV (Fig. 1a). In order to clarify further the association of viral proteins with virus structure, the external bilayer was solubilised with non-ionic detergent Trition X-100 and the dissociated components separated either by affinity chromatography or equilibrium centrifugation prior to PAGE analysis.

Triton X-100 and related compounds are mild detergents and most membrane enzymes retain activity in their presence. Helenius and Soderlund (1973) found that the envelope of Semliki Forest virus was solubilised by treatment with Triton X-100 in a step-wise manner which progressed with increasing concentrations of Triton X-100 from binding to, and disorganisation of, the lipid bilayer to disruption of the membrane with the formation of protein-lipid-detergent complexes. Complete solubilisation of the lipid was achieved at concentrations below 1%. Similar studies with vesicular stomatitis virus (Emerson and Wagner, 1972) have shown that removal of matrix protein from nucleocapsid structures is most effective at high salt concentrations.

MacNaughton et al. (1977) disrupted purified IBV with 1% Nonidet P40 in low salt buffer and separated virion components by sucrose density gradient centrifugation. Subsequent characterisation of the separated material failed to identify the presence of the 50,000 mol. wt. component in any fraction, although electron microscopy showed disrupted virions were penetrated by negative strain and apparently lacked the internal component. Similarly, Bingham and Almeida (1977) recovered only one band of protein at an average buoyant density of 1.28 g/cm^3 after treatment with Nonidet P40 and equilibrium centrifugation in a linear sucrose gradient. The continuing presence of membrane in the solubilised material obtained in both studies suggests that IBV particles were not completely solubilised under the conditions chosen by these workers.

Attempts were made in the present study to recover virion components after solubilisation with Triton X-100 by several different procedures. The use of affinity chromatography with immobilised concanavalin A has previously been shown to be effective in separating the solubilised components of hepatitis B surface antigen (Skelly et al. 1979).

Virus radiolabelled using the Bolton and Hunter reagent was used in this experiment. The presence of a 52,000 mol. wt. component in both fractions (Fig. 2) was an unexpected finding although the 29,000 mol. wt. glycoprotein was eluted only after application of α-methyl-D-mannoside. The fate of the slightly smaller, 26,000 mol. wt. glycopeptide was not clarified in these experiments.

The purification procedure performed as previously described (Lanser and Howard, 1980) allowed the production of unlabelled virus at high concentrations suitable for disruption with non-ionic detergents. Separation after Triton X-100 treatment by equilibrium centrifugation in sucrose gradients resulted in the recovery of two protein-containing bands. SDS-PAGE analysis showed that the band with a density in excess of 1.32 g/cm^3 contained a non-glycosylated polypeptide of mol. wt. 52,000 together with a glycopeptide of mol. wt. 26,000 (Figs. 5 and 6). Electron microscopy of this material revealed the presence of amorphous aggregates. The second band isolated at a buoyant density of 1.23 g/cm^3 contained both the 90,000 and 29,000 mol. wt. glycoprotein species. In addition, a non-glycosylated protein in the 52,000 mol. wt. range was also present (Figs. 5 and 6). The possibility therefore arises that there may be two non-glycosylated polypeptides migrating in the 52,000 mol. wt. range. The observation that no radiolabel sediments with 'core' structures after iodinated virus is solubilised together with unlabelled virus indicates that the Bolton and Hunter reagent does not penetrate these structures (data not shown). Therefore, the radiolabelled 52,000 mol. wt. structure eluted from concanavalin A by α-methyl-D-mannoside may represent an externally-situated component accessible to the Bolton and Hunter reagent which is joined non-covalently to either or both the 90,000 and the 29,000 mol. wt. glycoproteins. An alternative explanation is that Triton X-100 did not fully dissociate the virus under these experimental conditions. Further experiments such as tryptic peptide mapping of these two polypeptides would be necessary to determine whether they are indeed discrete viral proteins or Triton X-100 removes a proportion of the nucleocapsid protein which binds non-specifically with other components derived from the external lipid envelope.

The finding of a glycoprotein component in the denser band together with the major 'core' polypeptide is similar to observations reported for other coronaviruses, including haemagglutinating encephalomyelitis virus (Pocock and Garwes, 1977), transmissible gastroenteritis virus (Garwes et al., 1976), and mouse hepatitis virus (Wege et al., 1979). In each case, purified virus was disrupted by non-ionic detergents in low salt buffer and the component parts subsequently separated by centrifugation. Spherical structures were consistently isolated which contained RNA associated with a non-glycosylated polypeptide of approximately 50,000 mol. wt. In addition, at least one glycopeptide of 26,500 mol. wt. in haemagglutinating encephalitis virus and 24,000 mol. wt. in mouse hepatitis virus was present in these examples. Two glycopeptides have been found to be associated with the core of transmissible gastroenteritis virus with molecular weights of 30,000 and 28,500, respectively. Coronaviruses may therefore contain a complex nucleocapsid structure, with one or more glycosylated components.

The 90,000 and 29,000 mol. wt. glycopeptides of IBV are probably associated with

the lipid bilayer. The results of the present investigation do not indicate whether both are external to the envelope or whether one of them comprises the projections and the other is present as a translipid protein possibly attached to the internal 52,000 mol. wt. polypeptide. Further investigations will require development of suitable tissue culture systems for the metabolic radiolabelling of infectious bronchitis virus structural components and their precursors.

ACKNOWLEDGEMENTS

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