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THE PROTEIN COMPOSITION OF INFECTIOUS BRONCHITIS VIRUS

A THESIS SUBMITTED TO THE UNIVERSITY OF LONDON
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ABSTRACT

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The Protein Composition of Infectious Bronchitis Virus.

The Massachusetts strain of avian infectious bronchitis virus was purified from embryonated hens' eggs by polyethylene glycol 6,000 precipitation and isopycnic centrifugation through Metrizamide gradients. Four major polypeptides of apparent molecular weight 90,000, 52,000, 29,000 and 26,000 were resolved by SDS-polyacrylamide gel electrophoresis. Omission of reducing agent failed to resolve the 29,000 molecular weight component and increased the mobility of the 90,000 molecular weight polypeptide. Labelling of acrylamide gels with ¹²⁵I-concanavalin A indicated that polypeptides of molecular weight 90,000, 29,000 and 26,000 were glycosylated, and in the absence of reducing agent that the 29,000 glycopeptide migrated as a dimer in the 50,000 molecular weight region. Purified IBV radioiodinated with Bolton and Hunter reagent, which banded as a single peak of radioactivity in Metrizamide gradients, was found to contain bands of radioactivity when analysed by SDS-PAGE corresponding to the polypeptides of molecular weight 90,000, 52,000 and 29,000 resolved in stained gels. Disruption of IBV particles in Triton X-100 released two subviral particles; a 16 nm spike which was comprised of polypeptides with molecular weights of 90,000, 52,000 and 29,000; and another denser spherical particle 25-45 nm in diameter which contained nucleic acid and the 52,000 and 26,000 polypeptides. A model for the structure of IBV is presented.

CONTENTS

	Page
ABSTRACT	i
CONTENTS	ii
ILLUSTRATIVE MATERIAL	
INTRODUCTION	
A Classification and taxonomy of coronaviruses	1
B Ultrastructure	2
C Pathology and natural history	
1 Human coronaviruses	5
2 Murine coronaviruses	7
3 Swine coronaviruses	
Transmissible gastroenteritis	9
Haemagglutinating encephalomyelitis	10
4 Avian coronaviruses	
Infectious bronchitis	12
Coronavirus enteritis of turkeys	16
5 Other coronaviruses	
Rat coronavirus	17
Sialodacryoadenitis virus of rats	17
Neonatal calf diarrhoea	17
Feline infectious peritonitis	18
Canine coronavirus	19
D Coronavirus antigens and serology	19
E Physicochemical properties	
1 Protein composition	25
2 Genome	30

	Page
F Replication	40
G Tissue Culture Studies	41

MATERIALS AND METHODS

A Virus	44
B Estimation of virus infectivity	44
C Tissue culture methods	44
D Serological techniques	
1 Preparation of antisera	46
2 Complement fixation	47
3 Immunodiffusion	47
4 Radioimmunoprecipitation	47
5 Haemagglutination	47
E Virus purification	49
F Electron microscopy	50
G Electrophoretic procedures	50
H Radiolabelling procedures	52
I Chemical analyses	
1 Estimation of protein	52
2 Identification of glycoproteins	54
3 Treatment of virus with bromelain	55
4 Triton X-100 disruption of virus	55

RESULTS

A Virus growth in tissue culture	57
B Serological techniques	57
1 Immunodiffusion	57

	2	Complement fixation	57
	3	Neutralisation	58
	4	Haemagglutination	58
C		Virus purification	
	1	PEG precipitation	59
	2	Gel chromatography	59
	3	Centrifugation	60
D		Radiolabelling procedures	
	1	Chloramine-T Method	61
	2	Bolton and Hunter method	62
E		SDS-PAGE analysis of IBV	
	1	Effect of gel concentration	63
	2	Effect of reducing agents and temperature	64
F		Identification of IBV glycoproteins	65
G		Effect of bromelain on IBV	65
H		Triton X-100 disruption of IBV	
	1	Separation by Concanavalin A chromatography	67
	2	Separation by isopycnic centrifugation	63
		DISCUSSION	72
A		Purification	73
B		Polypeptide Analysis	77
C		Bromelain Treatment	83
D		Triton X-100 Solubilisation of Virus	38

	Page
CONCLUSION	96
ACKNOWLEDGEMENTS	99
REFERENCES	100
FIGURES	
TABLES	

ILLUSTRATIVE MATERIAL

TITLE

FIGURES

- 1 Electron microscopy of purified IBV.
- 2 Distribution of IBV complement-fixing antigen in a 5-45% Metrizamide gradient.
- 3 Gel chromatography of PEG precipitate from infected allantoic fluid through Biogel A-5m.
- 4 Gel chromatography of PEG precipitate from infected allantoic fluid through Sephadex G-75.
- 5 SDS-polyacrylamide slab gel electrophoresis of purified IBV in a 5-15% acrylamide gradient slab gel.
- 6 Gel chromatography of purified IBV radiolabelled by the Chloramine-T method
- 7 Sedimentation profile of ^{125}I -IBV in Metrizamide gradient.
- 8 SDS-polyacrylamide disc gel electrophoresis of purified IBV in 10% gels.
- 9 SDS-polyacrylamide disc gel electrophoresis of purified IBV in 7.5% gels.
- 10 SDS-polyacrylamide disc gel electrophoresis of ^{125}I -IBV solubilised in the presence of 2-mercaptoethanol.
- 11 SDS-polyacrylamide disc gel electrophoresis of ^{125}I -IBV solubilised in the absence of 2-mercaptoethanol.
- 12 SDS-polyacrylamide slab gel electrophoresis of purified IBV in 5-15% acrylamide gradient slab gel.
- 13 Identification of IBV glycoproteins.
- 14 Electron microscopy of purified IBV treated with bromelain.
- 15 SDS-polyacrylamide disc gel electrophoresis of purified IBV treated with bromelain.
- 16 SDS-polyacrylamide disc gel electrophoresis of purified IBV treated with bromelain.

- 17 Identification of glycoproteins in purified IBV treated with bromelain.
- 18 Affinity chromatography of Triton X-100 disrupted ^{125}I -IBV.
- 19 SDS-polyacrylamide disc gel electrophoresis of peak I obtained by Con A-Sepharose affinity chromatography of ^{125}I -IBV solubilised in Triton X-100.
- 20 SDS-polyacrylamide disc gel electrophoresis of peak II obtained by Con A-Sepharose affinity chromatography of ^{125}I -IBV solubilised in Triton X-100.
- 21 Sedimentation in a linear sucrose gradient of purified IBV solubilised in Triton X-100.
- 22 SDS-polyacrylamide disc gel electrophoresis of material banded at a density of 1.23 g cm^{-3} in a sucrose gradient containing IBV solubilised in Triton X-100.
- 23 Identification of glycopeptides in material banded at a density of 1.23 g cm^{-3} in a sucrose gradient containing IBV solubilised in Triton X-100.
- 24 Electron microscopy of material banded at a density of 1.23 g cm^{-3} in a sucrose gradient containing IBV solubilised in Triton X-100.
- 25 SDS-polyacrylamide disc gel electrophoresis of material pelleted to the bottom of a sucrose gradient containing IBV solubilised in Triton X-100.
- 26 Identification of glycopeptides in material pelleted to the bottom of a sucrose gradient containing IBV solubilised in Triton X-100.
- 27 Sedimentation in Metrizamide of radiolabelled and unlabelled IBV solubilised in Triton X-100.
- 28 Electron microscopy of band at a density of 1.22 g cm^{-3} in a Metrizamide gradient containing IBV solubilised in Triton X-100.

- 29 Electron microscopy of band at a density greater than 1.32 g cm^{-3} in Metrizamide gradient containing IBV solubilised in Triton X-100.
- 30 SDS-polyacrylamide disc gel electrophoresis of radiolabelled material at a density of 1.22 g cm^{-3} in Metrizamide gradients containing ^{125}I -IBV solubilised in Triton X-100.
- 31 Model of IBV structure.

TABLES

- 1 Coronaviruses of man and animals.
- 2 Polypeptides of Coronaviruses other than IBV.
- 3 Polypeptides of IBV.
- 4 Molecular weights of Coronavirus RNA.
- 5 Purification of IBV.

INTRODUCTION

Classification and Taxonomy of Coronaviruses

Avian infectious bronchitis virus (IBV) is the type species of a group of viruses which cause disease in a variety of animals (see Table 1.) and are placed together in one monogeneric family, the Coronaviridae, owing to their similar morphology as revealed by electron microscopy. In negatively stained preparations the virions appear as moderately pleomorphic enveloped particles 60-220 nm in diameter covered with a corona of distinctive club shaped projections 12-24 nm in length (see Fig. 1). Prior to 1968 viruses with this morphology had been tentatively placed with myxoviruses. However their morphology does differ particularly in the shape of the spikes which in myxoviruses appear rectangular and in coronaviruses are more bulbous with a very thin base where they are attached to the membrane. In 1968 an informal committee of virologists proposed that these viruses should constitute a new group and named the genus Coronavirus after the appearance of their layer of projections as seen in negatively stained preparations (Tyrrell et al, 1968). Since then studies on the biochemistry replication, structure and immunology of coronaviruses have justified their inclusion in a separate family (Tyrrell et al. 1975, 1978).

Coronaviruses possess a large single stranded genome with a covalently attached poly (A) sequence at the 3' terminus. RNA isolated from IBV and mouse hepatitis virus (MHV) is infectious and therefore the genome is thought to be of positive (messenger) polarity. Estimations of the molecular weight of coronavirus RNA have varied from $5.6 - 9.0 \times 10^6$ with corresponding sedimentation coefficients

varying from 48 - 70S. Replication occurs in the cytoplasm of infected cells and maturation is by budding through the endoplasmic reticulum.

The virion has a buoyant density of 1.16-1.23 g cm⁻³ in sucrose and 1.23-1.24 g cm⁻³ in CsCl. Estimates of sedimentation coefficient range from 330 to 495 S. Reports of the number and size of structural polypeptides show wide variation. From 7 to 16 peptides have been reported for IBV and between 4 and 7 for other members of the group. Most are glycopeptides (see Tables no. 23).

No subgroups in the family have been defined. However, Tyrrell et al. in their second report suggest that a tentative subgrouping may be made on serology.

The natural host range of the coronaviruses is generally restricted to the primary host species (see Table 1).

Ultrastructure

All coronaviruses have a similar external appearance when visualised by electron microscopy of negatively stained preparations. Davies and MacNaughton (1978) compared the morphology of three coronaviruses; mouse hepatitis virus (MHV), human coronavirus (HCV) strain 229E and infectious bronchitis virus (IBV). All coronaviruses examined were found to be pleomorphic and enveloped with a diameter of 80-200 nm surrounded by projections 18-22 nm in length. When negatively stained under normal conditions of preparation virions usually appeared hollow in the centre where stain collected. However if they were negatively stained with uranyl acetate, or previously freeze dried, they appeared circular without a central indentation.

Variations in the morphology of the projections of the corona have been reported. Davies and MacNaughton (1978) showed that the shape of the projections of MHV differed from those of HCV 229E and IBV. Intra-species variations were reported for IBV (Harkness and Bracewell (1974); the Beaudette and Connecticut strains appeared to lack projections whereas strain 927 carried only thin radiating projections with a small distal knob-like dilation in sharp contrast to the normal petal shaped projections of other IBV strains. There was no direct relationship between virulence and morphology, however those strains possessing a prominent corona induced high levels of group antibody.

The internal morphology of coronaviruses has yet to be characterized fully. In ultrathin sections coronaviruses have been shown to contain a distinct pair of electron dense shells, either in close apposition to each other in the case of IBV Beaudette strain (Becker et al, 1967) or separated by an electron lucent space as in MHV. (David-Ferreira and Manaker, 1965) and HCV 229E (Becker et al, 1967). In another study Apostolov et al (1970) looked at IBV particles in ultrathin sections and were able to visualise a thread-like internal component surrounded by a three layered unit membrane.

Two reports employing negative staining techniques suggest that the internal component of human coronaviruses consists of RNP in a continuous linear strand which may be coiled when packed within the virus (Kennedy and Johnson-Lussenburg, 1975, 1976, MacNaughton and Madge, 1978). MacNaughton and Madge (1978) also examined the internal architecture of MHV and found a single stranded helix of diameter 14-16 nm composed of subunits each with long axis 5-7 nm surrounding a hollow core of diameter 3-4 nm. These authors failed to visualise an internal component for IBV.

Other workers have used detergents to disrupt particles followed by centrifugation to separate components prior to electron microscopy. Treatment of HEV (Pocock and Garwes 1977), TGEV (Garwes et al, 1976) and MHV (Wege et al 1979) with NP40 resulted in the separation of fast sedimenting spherical particles which in TGEV were 20 nm in diameter less than untreated virus, and in HEV were the same diameter as untreated controls. No dimensions are given for the subviral particle isolated from MHV. The internal component could not be visualised in any of these spherical particles. It is suggested that these particles may be the inner shell observed in ultrathin sections of coronaviruses.

Recently Bingham and Almeida (1977) found that in situ treatment of purified IBV with NP40 on electron microscope grids resulted in a controlled breakdown of virions. The outer membrane was removed to reveal an internal membranous sac which in turn could be degraded with continued detergent treatment. From these observations they constructed a model of the IBV virion and postulated that it consists of (1) an outer membrane to which are attached the projections and (2) an inner membranous sac or flask which is attached to the outer membrane by the neck. These authors were unable to isolate a spherical subviral particle similar to those found in MHV, TGEV and HEV on disruption of IBV particles with NP40 followed by isopycnic centrifugation in sucrose density gradients. Instead they recovered material at a density of $1.27 - 1.28 \text{ g cm}^{-3}$ which was seen in electron micrographs as aggregates of amorphous membrane-like structures.

Pathology and Natural History

Human Coronaviruses

Coronaviruses pathogenic for man were first associated with respiratory infection (Tyrrell and Bynoe, 1965). A specimen collected from a school boy in 1960 induced cold symptoms in volunteers and the infection could be passaged in humans using infected nasal secretions. However no agent could be isolated using conventional tissue culture techniques. An infective agent was identified in nasal washings only by passage in vitro using organ cultures of human embryonic nasal or tracheal epithelium. There was no indication of viral growth in the organ cultures such as reduction in ciliary activity although interference did occur on challenge with other viruses such as Sendai and echovirus-11. The organ culture fluids collected from 1 day up to 8-days after inoculation produced colds in volunteers. This agent was called B814.

At the same time Hamre and Procknow (1966) reported the isolation of a similar virus from 5 medical students, 4 of whom had upper respiratory tract infections. These isolates were grown in human kidney cells but recognised only after a second blind passage. They were subsequently shown to replicate in human diploid cell lines with an accompanying cytopathic effect. After laboratory passage the agent produced common cold symptoms when inoculated into human volunteers. The prototype was named 229E and was suggested as being representative of those human coronaviruses which could be isolated in tissue culture. Of interest was the finding that the 229E strain was antigenically distinct from the B814 isolate.

The morphology of B814 and 229E was studied by electron microscopy by Almeida and Tyrrell (1967) using negatively stained preparations of lightly homogenised organ cultures. For the first time the similarity between these particles and those of avian infectious bronchitis virus was noted.

A further survey was performed by McIntosh et al, (1967) whereby specimens from adults from which no agent could be isolated by conventional tissue culture techniques were inoculated into human embryonic tracheal organ cultures. From 23 such specimens 6 produced agents with similar morphology to B814 and 229E. These agents, which could grow only in organ culture, were designated 'OC' strains to distinguish them from the 229E-like tissue culture strains from which they are antigenically distinct.

The respiratory tract infections caused by coronaviruses are generally mild and most prevalent during the winter months and early spring (Monto, 1974). Owing to the difficulty experienced in growing human coronaviruses most epidemiological data are based on serological studies. All age groups are susceptible to infection (McIntosh, 1974).

Recently coronaviruses have been associated with enteric disease in humans (Mathan et al. 1975, Caul and Clarke, 1975). Identification of the implicated agent as a coronavirus has been tentatively made solely on electron microscopic appearance of particles obtained from faeces. Caul and Clarke (1975) prepared a faecal emulsion and inoculated it into human embryo intestinal organ culture. After two days incubation the columnar epithelium of the villi became detached and electron microscopy showed the presence of large numbers of particles similar to those seen in the

faeces. The virus could not be passaged to any other in vitro system nor to another human intestinal organ culture. The specificity of this agent remains to be confirmed.

Furthermore, during a recent study in Western Australia Schnagel et al (1978) found coronavirus-like particles in faeces to be equally prevalent in people both with and without diarrhoea. They also found virus in the stools of a greater proportion of aboriginal than non-aboriginal children. The proportion of children excreting virus increased with age to 6 years. Interestingly, domestic dogs were also found to be excreting a virus similar in appearance.

Another human disease with which coronaviruses have been implicated is the progressive renal disease, endemic Balkan nephropathy (Apostolov and Spasic, 1975). Virus has not been isolated from affected kidneys however numerous cytoplasmic vesicles containing particles 80-200 nm resembling coronaviruses have been seen in thin sections of kidneys. Since the disease also occurs almost exclusively in people who have been in close contact with pigs which are natural hosts for coronaviruses Apostolov and Spasic (1975) suggest that a coronavirus may be the aetiological agent. Other agents including aflatoxins have been associated with the disease.

Murine Coronaviruses

Mouse hepatitis virus (MHV) causes both hepatitis and encephalitis in mice with different strains exhibiting tropism for one or the other (McIntosh, 1974). For example the strain designated JHM causes widespread destruction of myelin in the central nervous system. In contrast the A59 strain typically causes hepatitis with accompanying

necrosis of Kupffer and parenchymal cells. The virus also has the ability to destroy lymphoid cells in both spleen and bone marrow.

The virus was first recognised in a closed mouse colony (Gledhill and Andrews, 1951). Under these conditions it may become endemic, possibly because of the prevalence of antibody resulting in widespread subclinical infection but can be controlled by using caesarean derived barrier sustained stocks (McIntosh, 1974).

The pathogenicity of MHV is markedly enhanced by various forms of stress such as infestation with Eperythrozoon coccoides (Niven et al, 1952), simultaneous infection with other viruses (Chany and Robbe-Maridor, 1969) and pretreatment of mice with corticosteroids (Manso et al, 1959).

Different strains of mice show variation in susceptibility to infection with MHV as do the cell cultures derived from their tissues. C₃H mice are genetically resistant to MHV grown in Princeton (PRI) mice. This resistance resides in the macrophages into which the virus may penetrate but not undergo replication (Shif and Bang, 1970). Virelizier and Allison (1976) reported that the in vitro susceptibility to MHV 3 of peritoneal macrophages is closely correlated with the severity of the disease induced by the virus in vivo; C57BL, DBA/2, BALB/c and NZB strains of mice die of acute hepatitis whereas C₃H and A2G maintain a persistent infection with neurological symptoms.

The virus is found in the faeces of mice (Rowe et al, 1963) and is probably spread by the faecal-oral route. Rowe et al (1963) postulated it to be naturally a highly contagious enteric disease of varying severity with hepatitis if provoked in genetically susceptible mice.

The importance of understanding MHV infection lies in its usefulness as a model for studies on hepatitis and encephalitis. Recently Hierholzer et al (1973) described an isolate of MHV closely resembling MHV strain S which caused an epizootic of fatal diarrhoeal disease in ICR infant mice. Intestinal lesions included blunting of villi with syncytium formation, cytonecrosis and desquamation of epithelium. No lesions were found in other organs, and there was no evidence of virus in tissues other than intestinal epithelium. These workers suggest that enteritis in mice induced by MHV may be usefully studied as a model of diarrhoeal disease.

Swine Coronaviruses

Transmissible gastroenteritis virus (TGEV) causes a highly contagious enteric disease of swine accompanied by vomiting and severe diarrhoea. A high mortality results in piglets infected under 2 weeks of age. All ages are susceptible (Haelterman and Hutchings 1956) but in swine over 5 weeks of age mortality is low and clinical symptoms are limited to loss of appetite and diarrhoea for one to two days. It was first reported by Doyle and Hutchings (1946) in the United States and is now found worldwide (Bohl, 1975).

Infection occurs by ingestion of the virus which is resistant to low pH so remains viable until it comes in contact with the highly susceptible epithelial cells of the small intestine. Once these cells are infected they are rapidly destroyed or their function altered resulting in villous atrophy and an acute malabsorption syndrome (Haelterman, 1972). The ultimate cause of death is dehydration. Regeneration of villi may be seen after 4 to 5 days and is usually complete after 10 days (Hooper, 1972). The virus replicates in epithelial cells lining the jejunum and duodenum and ileum but not

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the stomach or colon. Replication in the respiratory tract also occurs (Hooper and Haelterman 1966a).

The disease is most frequently diagnosed and causes most loss at farrowing time (Haelterman and Hutchings 1956) and can spread rapidly through a susceptible herd so that in 3 days most animals are infected. Excretion of the virus may occur up to 3 weeks post infection but usually not after the faeces become firm (Hooper 1972). Introduction into a herd is postulated to occur when an infected animal is brought in or the virus is carried on contaminated boots from another farm (Bohl, 1975). Starlings can excrete the virus in their faeces and it is thought that this may be another mode of transmission from herd to herd (Pilchard 1965).

There is only one antigenic type of TGEV virus therefore vaccination of susceptible animals does not present the difficulty of antigenic variation which occurs with IBV. Immunity to TGEV does not correlate with the level of circulating antibody (Bohl et al., 1972). Protection is by ingestion of milk or colostrum containing antibody which neutralises the virus in the gut (Haelterman and Hutchings 1956). The ability to transmit immunity by the sow appears to be related to the level of IgA in the milk and this is only high enough if the gastrointestinal tract has been adequately stimulated antigenically. This will occur after a natural infection or vaccination with a live attenuated vaccine. Sows are able to transmit maternal antibody for 9-12 months post infection (Bohl et al., 1972).

Haemagglutinating encephalomyelitis virus of pigs

Haemagglutinating encephalomyelitis virus (HEV) also causes disease in swine. However, unlike TGEV, the disease it causes is characterized by two syndromes: a severe encephalitis with almost

100% fatality in piglets up to one week of age or a vomiting, wasting disease in piglets under two weeks of age with a lower mortality however the survivors are not commercially viable (Roe and Alexander 1958). The disease may also be known as vomiting and wasting disease of pigs to describe the second syndrome. Adults develop vomiting and anorexia but usually recover.

The disease was unrecognised before 1958 when it was reported from Ontario, Canada by Roe and Alexander (1958). The syndrome described in that outbreak was predominantly of the vomiting and wasting type without signs of nervous system involvement. The causative agent was not isolated. The following year Alexander et al (1959) described a further outbreak in the region which was characterized by severe encephalitis but little vomiting although the early clinical signs did include anorexia and constipation. The first isolation of the causative agent of the disease was made by Greig et al (1962).

The disease was first seen in England in 1958 (Cartwright et al 1969). The syndrome recognised then was of the vomiting and wasting disease type without overt nervous system involvement. The virus was isolated in pig kidney monolayers from brain suspensions of affected pigs and was shown to be identical serologically to strain BEV 21 isolated by Greig et al (1962).

The pathology of the disease also varies from that of TGEV. Cartwright and Lucas (1970) were unable to isolate the virus from the lower gastrointestinal tract using rectal swabs but did isolate it from throat swabs from 4-5 days post infection in experimentally infected piglets. Isolation was best achieved from the respiratory tract, tonsil, hind brain and spinal cord.

The virus is thought to be transmitted by infected nasal secretions to the respiratory tract where it replicates. That the virus cannot be isolated from the gastrointestinal tract but may be grown from the brain stem suggests that the signs of vomiting and wasting are centrally induced and the virus does not act locally on the gut. The virus is excreted mainly, if not only, by nasal excretions (Pensaert and Callebaut, 1971).

All strains are related serologically but there is no vaccine available. The disease in young pigs is controlled by exposing breeding sows to HEV two to three weeks before farrowing so that piglets are protected by colostral antibody.

Avian Coronaviruses

Avian infectious bronchitis disease was first described by Schalk and Hawn (1931) and was subsequently shown to be of viral aetiology (Beach and Schalm, 1936 . Beaudette and Hudson 1937). It occurs naturally only in the chicken and is a highly contagious, acute respiratory infection with a worldwide distribution. (Hofstad , 1972 , Cunningham 1975).

The natural infection is transmitted primarily by the aerosol route to the trachea where the virus multiplies rapidly. The incubation period varies from 18-36 hours after experimental inoculation to 36-48 hours for a natural infection. Virus can be isolated from trachea and lungs 24 hours post-infection and subsequently up to 8 days. A viraemia occurs after which the virus can be isolated in kidney, spleen, bursa and oviduct where it may persist for longer periods than in the respiratory tract (Hofstad 1972). Cook (1968) investigated carrier rates of the virus in chickens after infection. When

birds were kept in strict isolation IBV could be isolated from tracheal and cloacal swabs up to 49 days post infection. However when isolation was less effective the virus could be isolated more than 4 months later even though circulating antibody was present. In the latter case continued isolation of the virus was probably owing to continued reinfection from extraneous sources and no true carrier state occurred after IBV infection.

The virus is rapidly transmitted among chickens in a flock, with susceptible chickens showing symptoms 48 hours after being placed with infected birds. There is some evidence that farm to farm transmission by airborne droplet nuclei may occur. The disease is not spread by vectors but may be introduced indirectly by the use of contaminated utensils or feed (Cunningham 1975).

In chickens of all ages symptoms of the disease include tracheal rales, gasping and coughing. Young chicks may have a nasal discharge, wet eyes and swollen sinuses. They appear depressed and lose weight. Chickens infected when less than 2 weeks may suffer permanent damage to the oviduct. Mortality is rare in older birds. In adults the main economic loss is caused by a drop in egg production which is especially marked in laying flocks which contract the disease in the latter part of their laying year. These flocks may require a longer time to recover and their maintenance usually becomes uneconomic. The quality of the egg is also affected and the shell may be soft, rough and misshapen. Virus may be transmitted from oviduct to egg resulting in disease in the progeny but this seems to be of little importance to the epidemiology of the disease (Hofstad 1972).

In 1962 a new syndrome was described in the United States in which chickens developed nephrosis (Cosgrove, 1962). Winterfield and

Hitchner (1962) isolated two new strains, Gray and Holte, from chickens displaying similar clinical signs of mild respiratory involvement associated with serious nephritis and nephrosis. The new strains differed antigenically from the common strains; Massachusetts, Connecticut and Iowa. In the same year Cumming (1962) reported a similar syndrome to be present in Australian flocks and also caused by IBV.

Three weeks after natural infection a high level of antibody is obtained, and this persists for about a year to give resistance to infection by homologous strains. However after this time reinfection may occur. Local tracheal immunity seems to be important. Maternal antibody is passed to the chick and is highest immediately after hatching, declining to negligible levels by four weeks of age. Maternal antibody reduces the severity of the disease but will not prevent it (Hofstad, 1972).

The disease is diagnosed by demonstrating ascending antibody titre. Clinically infectious bronchitis is very difficult to distinguish from Newcastle Disease and laryngotracheitis (Hofstad, 1972). To demonstrate the virus in sick birds a broth suspension of lung and trachea is made during the early stages of the disease and inoculated into the trachea of a susceptible chicken. The experimental disease should be apparent in 18 to 36 hours. Virus can be isolated in chicken embryos which are inoculated when 9 to 11 days old and its presence demonstrated by inoculating allantoic fluid after 48 to 96 hours into susceptible birds (Hofstad, 1972).

Vaccination has been used to control the disease in flocks. Two types of vaccine have been tested: inactivated virus vaccines which are not very effective in preventing infection with challenge strains,

and modified live virus vaccines which have been found to be effective and are in use. Strains used in the latter type of vaccine have usually been passed in chicken embryos at least 25 times to reduce their pathogenicity. These strains multiply in the mucosa of the trachea where infection is restricted to the subepithelial cells. In contrast, a naturally occurring virus infects also the surface epithelium of the respiratory tract causing necrosis of the cilia and ultimately a viraemia. The number of passages necessary to modify the pathogenicity varies from strain to strain and there may be concomittant loss in immunogenicity. Vaccine strains may however still cause symptoms in very young chicks if they are not protected by maternal antibody (Hofstad , 1972).

Many serotypes of IBV are recognised by neutralisation tests and chicken immunity and protection tests. These include Massachusetts (IBV 41), Connecticut (IBV 46), Iowa 97, Iowa 609, SE 17, Clark 333, Gray, Holte, J.M.K., Cuxhaven and Australian T. Indicator systems used for neutralisation tests have included chicken embryo, chick embryo kidney tissue culture and chick kidney cell culture in which a plaque reduction test has been developed. However difficulty arises with the use of these cell cultures because not all isolates of IBV can be adapted to grow in chicken kidney cells.

Since there are several serotypes of IBV the vaccine strain should have a broad range of antigenic components. The Massachusetts strain has been found to give optimum immunity against heterologous strains (Hofstad , 1961; Winterfield, 1968). The use of more than one strain in a single combined vaccine is associated with a greater risk of prolonged respiratory reaction. It has been shown that inclusion of the Massachusetts strain will interfere with the

and modified live virus vaccines which have been found to be effective and are in use. Strains used in the latter type of vaccine have usually been passed in chicken embryos at least 25 times to reduce their pathogenicity. These strains multiply in the mucosa of the trachea where infection is restricted to the subepithelial cells. In contrast, a naturally occurring virus infects also the surface epithelium of the respiratory tract causing necrosis of the cilia and ultimately a viraemia. The number of passages necessary to modify the pathogenicity varies from strain to strain and there may be concomittant loss in immunogenicity. Vaccine strains may however still cause symptoms in very young chicks if they are not protected by maternal antibody (Hofstad , 1972).

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development of antibodies to the Connecticut strain (Winterfield, 1968).

Maternal antibodies do not materially interfere with the immune response to live virus vaccine as judged by challenge and in most flocks they are present in chicks (Raggi and Lee, 1965). Therefore vaccination of chicks a few days after hatching is both expedient and safe. However, an optimum immune response is usually obtained by vaccination at 6 weeks (Hofstad 1972).

Coronavirus enteritis of turkeys (CET) Bluecomb disease

Bluecomb is an acute intestinal disease of turkeys characterized by dehydration, loss of appetite, loss of weight and wet droppings. All ages are susceptible but there is a higher morbidity and mortality in young turkeys 1 to 6 weeks of age (Nagi et al., 1975). Serious outbreaks had been reported since 1951 in the United States, especially in the Minnesota region (Pomeroy, 1978), and various agents had been associated with the disease. A coronavirus, however is now considered to be the causal agent (Nagi et al 1975, Desbmukh and Pomeroy, 1974). The virus has not been reported to grow in tissue culture but has been isolated in the intestines of 24 day old turkey embryos inoculated by the amniotic route (Desbmukh and Pomeroy 1974). Identification of the virus has hitherto been on morphological criteria no serological relationship with other coronaviruses having been reported (Pomeroy, 1978).

The disease is spread by the faecal-oral route and spreads rapidly throughout a susceptible flock. There is a 2-3 day incubation period and illness may last from 10 days to 2 weeks. Unlike IBV infection in chickens the infection is confined to the gastrointestinal

tract and the virus may be excreted for several months after infection. There is no vaccine available (Pomeroy, 1978).

Other Coronaviruses

Rat coronaviruses

Rat coronavirus (RCV) causes a fatal pneumonitis in newborn suckling rats and assymptomatic pulmonary infection in adults. The disease is very widespread in colonies but was not isolated until 1970 (Parker et al. 1970), although there was serological evidence that a virus antigenically related to MHV did cause disease in rats (Hartley et al. 1964).

Sialodacryoadenitis Virus of Rats

In 1969 Jonas et al reported the isolation of a viral agent which caused sialodacryoadenitis in rats and demonstrated virus-like particles in thin sections of salivary glands of experimentally infected germ free rats. Subsequently the virus was shown to be antigenically related to MHV and to the rat coronavirus described by Parker et al. (1970) and to possess all the biological characteristics of coronaviruses (Bhatt et al., 1972).

Neonatal calf diarrhoea coronavirus (NCDCV)

Various viruses are known to cause enteritis in cattle and in 1972 a coronavirus was also implicated as a primary pathogen causing diarrhoea in calves (Stair et al., 1972). Faecal material from infected animals was filtered to remove bacteria and fed to gnotobiotic and normal calves both of which developed diarrhoea. The faecal material was subjected to sucrose density centrifugation and a virus recovered

which had the typical coronavirus morphology. No other viruses were recovered from the material. The following year a report was published of the isolation of the virus in primary foetal bovine kidney (FBK) tissue culture and was subsequently adapted to secondary FBK cells (Mebus et al., 1973). The characterization of NCDCV as a coronavirus was made in 1976 by Sharpee et al.

The virus causes a disease in cattle very similar to that caused by TGEV in swine except that the epithelial cells of the intestinal villi and colon are predominantly affected (Mebus et al., 1973). NCDCV, attenuated by serial passage in FBK tissue culture, has been used successfully as a live vaccine (Mebus et al., 1973).

Feline infectious peritonitis virus

Feline infectious peritonitis (FIP) occurs in cats of all ages and is characterized by gradual abdominal distension with progressive emaciation. Once these symptoms are evident cats usually die within one to 8 weeks. Neurological and ocular involvement may occur (Horzinek and Osterhaus, 1979). The mode of transmission is unknown. The incubation period may extend up to 4 months and animals may survive for a further 6 months. The chronic nature of the disease and its clinical manifestations, suggest that FIP may have an immune complex pathogenesis (Horzinek and Osterhaus, 1979). A concurrent feline leukemia virus infection appears to enhance FIP symptoms. Similar enhancement of infection by oncoviruses is seen with MHV infections in mice.

Canine Coronavirus

Gastroenteritis in neonatal dogs and puppies associated with infection by a coronavirus-like agent was first reported in 1972 by Cartwright and Lucas (1972). They were unable to isolate a virus although they did notice a rapid rise in TGEV neutralising antibodies in the sera of infected dogs indicating that a virus closely related to TGEV may have been the causal agent. In a later report Binn et al., (1975) described the isolation of the agent and established that coronaviruses could be the primary agent of gastroenteritis in dogs.

The pathogenesis of the disease is very similar to that of TGEV in swine and NCDCV in calves. The virus infects the absorptive epithelium of the small intestine to cause villus atrophy and acute diarrhoea. The end of the diarrhoeal phase coincides with recovery of the small intestine mucosa (Keenan et al., 1976). The incubation period is very short and illness is self limiting with the development of antibodies. The disease is not normally fatal but weak puppies may succumb (Cartwright and Lucas, 1972). The virus causes a severe disease in very young puppies because of general intestinal immaturity although it may be confined to the immature ileum in older animals. These characteristics of canine coronavirus infection in dogs differ from coronavirus-associated enteritis in calves and swine; in TGEV infection the jejunum is most infected and with NCDCV infection the colon is predominantly infected (Keenan et al., 1976).

Coronavirus Antigens and Serology

Techniques that have been used to study the intra and interspecies antigenic relationships among coronaviruses include virus neutralisation, immunodiffusion, counter immunoelectrophoresis, complement

fixation, immunofluorescence and haemagglutination. The number of distinct antigenic molecules associated with the virions of coronaviruses have been studied mainly by immunodiffusion and counter immunoelectrophoresis tests.

Hierholzer et al. (1972) found at least 3 antigens associated with the OC 43 strain of human coronaviruses but were unable to determine precisely the number as monitored by immunodiffusion tests. It was suggested that this particular difficulty may have been related to the inadequate disruption of the virus. Hierholzer et al used virus purified from suckling mouse brain as immunogen for both guinea pigs and chickens. Analysis of the antibody responses indicated that an antigen present in normal mouse brain reacted with hyperimmune sera from these animals. They suggest from this evidence that OC 43 virus contains incorporated host material.

Bradburne (1970) reported 2 antigens to be present in mouse hepatitis virus (MHV 3) by immunodiffusion tests. The serum was obtained from mice infected with the virus. Three antigens have been detected in the swine coronaviruses, TGEV (Bohac et al., 1975) and HCV (Mengeling, 1972) by immunodiffusion against homotypic hyperimmune sera.

One of the early experiments with IBV antigens was performed by Tevethia and Cunningham (1968). Three antigens were released from the IBV virion by disruption with ether, one of which was identified as a ribonucleoprotein by its susceptibility to RNase and 10% acetic acid. Immunodiffusion was used to detect the antigen/antibody reactions.

Serological evidence that IBV contains host material is given in a study by Berry and Almeida (1968). They demonstrated that heterotypic and homotypic hyperimmune sera neutralised IBV significantly when unheated. By electronmicroscopy antibody could be seen attached to both the surface projections and virus envelope, and if unheated, this antiserum was also associated with the production of holes approximately 10 nm in diameter in the lipid bilayer. Unheated rabbit serum prepared against uninfected chicken embryo fibroblasts also contained antibody which was associated with the production of similar holes in IBV envelopes. Antibody from heated anti-chick embryo serum could be seen attached to the envelope only and was not associated with holes. Unheated and heated anti-chick embryo sera also contained neutralising antibody to the virus.

The ability to produce holes in the membrane was restored to heated rabbit antisera by the addition of either unheated control rabbit serum or normal unheated guinea pig serum. Homotypic antisera, both heated and unheated, contained antibody which was seen to attach only to the surface projections of the virus: no attachment to the membrane was visible.

Berry and Almeida give this as evidence that the heterologous species 'can recognise membrane components of IBV grown in eggs as being foreign but not so the chicken, the homologous species'.

Few studies have attempted the separation of virion components followed by their biochemical and antigenic characterization. Yaseen and Johnson-Lussenburg (1978) isolated the internal component of HCV 229E by disrupting viral particles with sodium deoxycholate or Triton X-100 followed by sucrose gradient centrifugation. The

internal component was found to be immunogenic in guinea pigs. In addition it reacted with human convalescent sera in cellulose acetate immunodiffusion tests. This 229E nucleoprotein was also found to react with TGEV, HEV and MHV antisera thereby showing that the internal components of these four coronaviruses possess at least one common antigen.

Garves et al. (1976) purified surface projections of TGEV by solubilising the virus in Nonidet NP40, separating virion components by rate zonal centrifugation and precipitating the protein of the projections with ammonium sulphate. The inoculation of this preparation into pregnant sows resulted in the production of neutralising antibodies to TGEV.

Not all coronaviruses agglutinate red cells. Human strains OC38 and OC 43 agglutinate chicken, rat and mouse erythrocytes at all temperatures and human O and vervet green monkey cells at 4°C only. HEV haemagglutinates only rat, chicken, turkey, mouse and hamster cells at 22°C (McIntosh, 1974). NCDCV agglutinates a range of cells similar to HEV except avian red cells. Haemadsorption also occurs with these viruses owing to the presence on the surface of infected monolayers of adherent virus particles; this is in contrast to haemadsorption in myxovirus infected cells where red cells adhere to maturing virus at the plasma membrane (Bucknall et al., 1972).

Infectious bronchitis virus has been reported to possess haemagglutinating activity after treatment with enzymes. Corbo and Cunningham (1959) demonstrated that virus particles modified by trypsin treatment were able to agglutinate chicken erythrocytes but this reaction could be inhibited by both anti-IBV and normal sera.

Bingham et al (1975) demonstrated haemagglutination activity in IBV preparations which had been previously treated with phospholipase C. This activity was inhibited by specific antisera. However not all strains of IBV appear to haemagglutinate erythrocytes after enzyme treatment (Alexander and Chettle, 1977; Alexander et al. 1976). A haemagglutination inhibition test has been developed using phospholipase C treated virus for the detection of IBV antibody in infected chickens (Alexander and Chettle 1977).

Data on serological relationships amongst the Coronaviridae are incomplete owing in part to the difficulty experienced in growing these viruses and in part a lack of knowledge of the specificity of different antigens. Different strains amongst coronaviruses infecting each host species are recognised by serum neutralisation tests. All isolates of TGEV are antigenically identical as indeed are all isolates of HEV. However this appears to be a unique occurrence among the coronaviruses (McIntosh 1974). Human coronaviruses grown in tissue culture are found to constitute a homogeneous group which do not cross react with organ culture strains (McIntosh et al., 1969) although OC 43 antisera neutralises 229E (Bradburne, 1970).

There have been two comprehensive studies on the relationships among different coronaviruses (McIntosh et al., 1969, Bradburne 1970). The studies however, are incomplete in that only human, mouse and IBV strain Beaudette were examined. Both indicated that IBV appeared unrelated to any other coronavirus by complement fixation, neutralisation, fluorescent antibody and immunodiffusion tests. Strong cross reactions were revealed between human strains grown in organ culture and MHV.

Later serological studies have been fragmentary and reports of antigenic relationships among presently recognised members of the group are confusing. The three rodent coronaviruses; MHV, rat coronavirus and sialodacryoadenitis virus of rats are inter-related by neutralisation and complement fixation tests (Bhatt et al. 1972) and also cross react with some human strains (Hartley et al. 1964).

The two porcine coronaviruses are not closely related antigenically. However TGEV is neutralised by sera from feline infectious peritonitis (FIP) infected cats (Reynolds et al. 1977). FIPV has also been shown to cross react with canine coronavirus and the human strain 229E (Horzinek and Osterhaus, 1979).

Recently the antigenic relationships between 8 mammalian coronaviruses was studied using the direct and indirect fluorescent antibody procedures as the only serological test (Pedersen et al. 1978). Two groups of antigenically related viruses were defined. The first group comprised MHV 3, HEV-67N, NCDCV and HCV-OC43. The other group comprised FIPV, TGEV, HCV-229E and CCV. The avian coronaviruses were not included in the survey.

Physico-chemical Properties

Protein Composition

Analyses of the protein composition of mammalian coronaviruses have shown similarities to one another (see Table 2). All except two (Hierholzer et al. 1972, Hajer and Storz 1979) were on virus which had been grown in tissue culture and concentrated using techniques which did not involve high speed pelleting. The initial concentration step was by polyethylene glycol precipitation of human coronavirus 229E (Hierholzer 1970) and MHV (Sturman 1977, Wege et al. 1979) or ammonium sulphate precipitation of TGEV (Garwes and Pocock, 1975) and HEV (Pocock and Garwes, 1977). Hierholzer et al. (1972) purified human coronavirus OC43 from suckling mouse brain by extensive temperature dependant adsorption to human 'O' erythrocytes followed by pelleting. Hajer and Storz (1979) purified the enteropathogenic bovine coronavirus strain LY-130 from the intestinal contents of infected calves by sequential differential and velocity gradient centrifugation. All used isopycnic centrifugation as the final purification step. As in studies with IBV, the techniques used for sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of polypeptides varied among laboratories.

Hierholzer et al. (1972) purified a human coronavirus strain OC43 from suckling mouse brain by extensive temperature dependant adsorption to human 'O' erythrocytes followed by differential centrifugation. Six polypeptides with molecular weights of 191,000, 104,000, 60,000, 47,000, 30,000 and 15,000 respectively were revealed by 7.5% polyacrylamide gel electrophoresis in pH 7.2 phosphate buffer system. The purified virus was prepared for PAGE in 4% SDS and 2% 2-mercaptoethanol with 1.0 M urea for 1 hour at 37°C, then

100°C for 1 minute.

Hierholzer (1976) subsequently examined another human coronavirus 229E. Virus was grown in human embryonic fibroblast cultures and concentrated by either adsorption to CaHPO_4 gel, or polyethyleneglycol 6,000 precipitation. The virus was further purified by banding in 20-60% sucrose gradients or glycerol tartrate gradients. Analyses of polypeptide composition were performed by both the discontinuous tris buffer and continuous phosphate buffer systems of polyacrylamide gel electrophoresis in 7.5% and 8% gels. Similar results were obtained regardless of the gel system employed. No information was presented as to the preparation of the sample subjected to PAGE. Seven polypeptides were separated with molecular weights of 196,000, 165,000, 105,500, 65,500, 47,300, 31,400 and 16,900 respectively (Table 2).

Sturman (1977) reported the first analysis of the polypeptide structure of a murine coronavirus, mouse hepatitis virus, strain A 59. Labelled virus was grown in transformed mouse cells and the polypeptides in purified virions analysed using the discontinuous tris buffer PAGE system in 7.5% and 8% gels. These results were compared with those obtained using the neutral pH-phosphate gel system of Weber and Osborn (1975) and the neutral pH-phosphate urea gel system of Uemura and Mizisham (1975). Sturman discovered that the number and proportion of the polypeptides which were resolved varied with the method of preparation of the sample before being applied to the gels but did not vary with the SDS-PAGE system used. Four native polypeptides were resolved of molecular weight 180,000, 90,000, 50,000 and 23,000. After heating at 100°C in SDS, GP23 aggregated to produce polypeptides of much lower mobility and also several new forms of higher mobility,

approximately 60,000 and 38,000. The effects of heating were exaggerated by 2-mercaptoethanol and dithiothreitol. Sturman suggests from this experience that the variations in reported molecular weights of polypeptides comprising coronavirus virions may be owing to differences in sample preparation technique.

Another report of the structural polypeptides of a murine coronavirus was published by Wege et al (1979) using the JHM strain which is neurotropic. The virus was grown in Sac(-) cells, a permanent rhabdomyosarcoma line from mice, in the presence of ^3H -amino acids and ^3H -glucosamine, and subsequently purified by PEG 6,000 precipitation followed by pelleting through a 5-20% sucrose gradient onto a 60% sucrose cushion. The virus was then centrifuged to equilibrium in a 20-60% sucrose gradient. The discontinuous SDS-PAGE system of Laemmli (1970) was used to analyse the polypeptide composition of JHM. Samples were solubilised in 0.06 M Tris-HCl containing 2% SDS and 0.1% 2-mercaptoethanol and heated for 5 minutes at 56°C. Six polypeptides were resolved in labelled virus with molecular weights of 170,000, 125,000, 97,500, 60,800, 24,800 and 22,700. The molecular weights of polypeptides identified as glycopeptides by ^3H -glucosamine labelling were 170,000, 125,000, 97,500 and 24,800 respectively. Only polypeptides with molecular weights of 60,800 and 22,700 were non-glycosylated.

Wege et al. (1979) also compared the SDS-PAGE profiles obtained with different systems and found that the buffer used did not influence their results but these were affected by the method of sample preparation. They discovered that boiling the virus in the presence of urea and 2-mercaptoethanol led to strong aggregation of protein at the top of the gels, a decrease in three polypeptides and the

disappearance of the 125,000 molecular weight glycopeptide. These results were very similar to those reported by Sturman (1977) for MHV strain A59.

The polypeptide structure of TGEV has been examined by Garwes and Pocock (1975). The virus was purified from primary pig kidney cell culture fluid by precipitation with sodium sulphate followed by equilibrium centrifugation in linear sucrose gradients. The purified virus was then concentrated and prepared for analysis by PAGE. After heating at 100°C for two minutes in 1% SDS and 1% 2-mercaptoethanol, the polypeptides were separated by PAGE in 5% gels using a 0.1 M phosphate buffer system. Four major polypeptides were obtained with molecular weights of 200,000, 50,000, 30,000 and 28,500, as well as two minor polypeptides; 105,000 and 80,500.

Similar studies were performed on haemagglutinating encephalomyelitis virus of pigs (HEV) by the same group (Pocock and Garwes, 1977, Pocock 1978). HEV was grown in adult pig thyroid cells and purified in the same way as TGEV by first concentrating the virus with 40% saturated ammonium sulphate, then banding in two sucrose density gradients and finally pelleting to concentrate further. The polypeptide composition was analysed by PAGE using 5% gels in phosphate buffer 0.1 M pH 7.2. Virus was prepared for PAGE by heating at 100°C in 1% SDS and 1% 2-mercaptoethanol. Five polypeptides were resolved 180,000, 125,000, 100,000, 56,000 and 26,500. Only the polypeptide of molecular weight 56,000, was not glycosylated.

Purified HEV was treated with dithiothreitol to determine if disulphide bonds have any biological activity in the virion. Pocock was able to demonstrate that treatment with dithiothreitol removed the infectivity of the virus and reduced its HA activity at the same rate

as GP 125,000 was removed from the polypeptide profile. Up to 70% of GP 100,000 could also be removed by high concentration of dithiothreitol. Polypeptides 130,000, 56,000 and 26,500 were unaffected. This evidence suggests that GP 125,000 may be the important polypeptide in the haemagglutinin. No differences in the PAGE profiles of whole virions were observed when dithiothreitol or 2-mercaptoethanol were present in or absent from the sample preparation mixture prior to PAGE, suggesting that interpeptide disulphide bonds are not important to HEV virion structure. 2-mercaptoethanol but not dithiothreitol slightly affected the migration rate of 130,000 molecular weights component suggesting that an interpeptide disulphide bond may be present.

The protein structure of the enteropathogenic bovine coronavirus strain LY-138 was examined by Hajer and Storz (1979). This strain could not be adapted to tissue culture. Therefore virus for biochemical analysis was purified from the contents of the small intestine obtained from experimentally infected calves 3 days after oral inoculation. The initial purification step was differential centrifugation and subsequent steps included velocity and isopycnic centrifugation in sucrose and CsCl gradients. Intact virions had a buoyant density of 1.185 g cm^{-3} in sucrose. SDS-PAGE was performed in neutral sodium phosphate gels containing 0.1% SDS and 0.5 M urea. Samples for electrophoresis were solubilised in 1% SDS, 1% 2-mercaptoethanol and 2 M urea and boiled for three minutes. At least seven polypeptides were resolved with molecular weights of: 110,000, 100,000, 82,000, 70,000, 53,000, 45,000 and 36,000. Periodate-Schiff staining of gels revealed that four of these were glycoproteins with molecular weights of 110,000, 100,000, 53,000 and 45,000. This profile differs substantially from those reported

for other coronaviruses (see Table 2).

Attempts have been made to assign individual polypeptides to their positions on the coronavirus virion. Two approaches have been adopted: protease digestion of external proteins on intact particles or detergent disruption of particles followed by direct visualisation of their components with the electron microscopy or separation of components prior to analysis by SDS-PAGE and electron microscopy.

The effect of bromelain digestion on purified virions of the human coronavirus OC43 was examined by Hierholzer et al. (1972). Enzyme treatment was found to remove the outer projections of the corona with the concomitant loss of two glycoproteins of molecular weight 104,000 and 15,000. These two components were therefore thought to be major components of the outer projections. In the later study on another human coronavirus strain 229E Hierholzer (1976) found that bromelain removed the projections and the glycoproteins 105,000 and 16,900 which results were similar to those found for strain OC43.

There has been only one report of the disruption of a human coronavirus by non-ionic detergent treatment. Kennedy and Johnson-Lussenburg (1976) disrupted human coronavirus 229E in Nonidet P40 and then separated components by isopycnic centrifugation in sucrose gradients. A fast sedimenting particle with a buoyant density of 1.27 g cm^{-3} was obtained which was visualised by electron microscopy as a loosely twisted, helical, continuous strand with an average width of 9 nm. The authors suggest that these structures are nucleocapsids in which the RNP complex is uncoiling due to the physicochemical conditions of the disruption. They did not analyse this material by PAGE and therefore do not report which polypeptides were associated with the particles.

Sturman (1977) examined the sensitivity of the proteins of MHV strain A59 to bromelain and pronase and found that GP180 and GP90 were completely sensitive to these proteases, GP23 was 20% sensitive and GP50 completely resistant. Sturman suggested that (1) GP180 and GP90 comprise the petals of the corona, (2) the protease sensitive portion of GP23 lies outside the membrane, (3) the resistant portion is protected by it, and (4) that VP50 is the core protein.

The treatment of whole virions with trypsin resulted in no significant loss of label but there was more GP90 than GP180 in PAGE polypeptide profiles compared with untreated virions. Also tryptic digests of GP180 and GP90 gave identical peptide patterns. This provides evidence that GP90 is a cleavage product of the larger GP180 component.

When similar experiments were performed on the JHM strain of MHV (Wege et al. 1979) pronase at low concentration increased the mobility of GP1 from 170,000 to 150,000 suggesting that this glycopeptide may be only partially accessible to the enzyme and thus may be protected by the envelope. Further treatment with pronase removed the projections and glycoproteins of molecular weight 125,000 and 97,500 suggesting that they are components of the spikes. At higher concentrations the glycoprotein of molecular weight 24,800 was also reduced and the two non-glycosylated polypeptides minimally affected, suggesting that these three are inside the envelope.

Disruption of intact particles of JHM by Nonidet P40 released a fast sedimenting subviral particle of buoyant density 1.26 g cm^{-3} in sucrose. Electron microscopy of this material revealed spherical particles lacking the projections. SDS-PAGE resolved only three polypeptides; the non-glycosylated polypeptides of molecular weight

60,600 and 22,700 and the glycoprotein of molecular weight 24,800 suggesting that these three comprise the core of JHM located within the lipid bilayer.

Comparing the results obtained from both enzyme and non-ionic detergent treatment of JHM Wege et al. (1979) suggest that since the small glycopeptide of molecular weight 24,800 is inside the lipid bilayer attached to the non-glycosylated peptides but is still slightly affected by pronase, that it may be a transmembrane glycopeptide. Sturman (1977) from protease digestion experiments alone similarly suggests that the small glycoprotein of molecular weight 29,500 found in the A59 strain of MHV is also a transmembrane protein. In a later report (Sturman, 1978) the three glycoproteins could be separated from the non-glycosylated polypeptide of 50,000 molecular weight by Nonidet P40 treatment at room temperature followed by sucrose density centrifugation. When disruption was carried out at 37°C, however, the small glycoprotein of 23,000 molecular weight sedimented with the 50,000 molecular weight component, the core protein. Thus with A59 at least, the association of a small glycoprotein with the core non-glycosylated protein is temperature dependent. Similar temperature dependence was not reported for JHM by Wege et al. (1979) who performed all Nonidet P40 disruption experiments at 20°C.

Similar enzyme and detergent studies have been reported for TGEV and HEV. Bromelain treatment of purified TGEV virions resulted in the loss of the polypeptide of molecular weight 200,000 suggesting that the corona (petals) contain this polypeptide (Garwes and Pocock, 1975).

In a later publication Garwes, Pocock and Pike (1975) reported the effect of Nonidet P40 on purified TGEV virions. Nonidet P40 removed up to 98% of the lipid, degrading virus to two subviral particles which were then separated by sucrose rate zonal centrifugation and the resolved components characterized by both electron microscopy and polyacrylamide gel electrophoresis. The faster sedimenting band contained polypeptides 50,000, 30,000 and 28,500; contained RNA and had a sedimentation coefficient of 650S compared with intact virions which sediment at 495S. Although RNA was present, infectivity of this band could not be demonstrated. Electron micrographs revealed round particles resembling the cores of type B and C coronaviruses. No internal morphology could be distinguished and spikes were absent. The slower sedimenting protein band contained the 200,000 molecular weight polypeptide and was able to induce the formation of TGEV virus neutralising antibody when inoculated into pregnant sows. This data again suggests that the 200,000 molecular weight glycopeptide comprises the spikes of the corona.

Nonidet P40 treatment of purified HEV virions (Pocock and Garwes, 1977) released two forms of subviral particles, one of which sedimented in sucrose density gradients at the same rate as intact virions but contained no HA activity, and was not infectious. Projections were absent as demonstrated by electron microscopy. SDS-PAGE analysis of these spherical particles resolved the polypeptide of 56,000 molecular weight and the glycopeptide of 26,500 molecular weight. The other band sedimented more slowly, retained HA activity and contained the glycopeptides of 180,000, 125,000 and 100,000 molecular weight. These results suggest that in HEV these three peptides are either associated with the membrane or are externally situated with respect to the lipid bilayer.

A non-glycosylated polypeptide with a molecular weight of between 50,000 and 60,000 was consistently present in the above-mentioned reports. This component could be separated from the virion by disruption of whole particles with non-ionic detergents such as Nonidet P40 followed by isopycnic centrifugation. It was shown to be associated with fast sedimenting spherical particles which also contained RNA and at least one other smaller polypeptide one of which was glycosylated in MHV (Sturman, 1978, Wege et al. 1979), TGEV (Garwes et al. 1976) and HEV (Pocock and Garwes, 1977). In MHV, TGEV and human coronaviruses a component in this size range was not affected by protease digestion of whole purified virions. (The affect of protease treatment on HEV virions has not been reported.) From this evidence most workers agree that the 50-60,000 molecular weight component is the ribonucleoprotein.

The position of other polypeptide components on the coronavirus virion remains in doubt. In MHV A59 strain, TGEV and HEV the largest glycoprotein in the size range 180,000 to 200,000 may be associated with spikes of the corona. In MHV and TGEV this glycoprotein may be the only polypeptide comprising the projections.

There has been only one report of an attempt to identify the polypeptide associated with haemagglutinating activity in coronaviruses. Pocock and Garwes (1977) removed a glycopeptide of 125,000 molecular weight by Nonidet P40 disruption of HEV and found that it sedimented with polypeptides 180,000 and 100,000 as a band separate from the core. They found that this peptide could be removed from the polypeptide profile by treatment of whole virions with dithiothreitol and in parallel the haemagglutinating activity was reduced. From

this they suggest that the 125,000 molecular weight glycopeptide may be important in the haemagglutinin.

Investigations into the protein structure of the enteropathogenic bovine coronavirus (Hajer and Storz, 1979) produced results which were very different from those reported for other mammalian coronaviruses. No bands were detected in isopycnic sucrose or CsCl gradients onto which had been placed virions disrupted by Tween-80, Nonidet P40 or diethyl ether. The authors suggest that this is owing to virions being completely disassembled by these compounds. When virions were treated with chloroform and similarly processed however, core like structures 80 nm in diameter were found at a buoyant density of 1.201 g cm^{-3} in sucrose. These particles contained only non-glycosylated polypeptides of molecular weight 82,000 and 36,000.

Analyses as to the type and number of polypeptides contained in the IBV virion have come from only two laboratories in recent years (Bingham 1975, Collins et al. 1976, Alexander and Collins 1977, MacNaughton and Madge 1977, MacNaughton et al. 1977). All but one (Alexander and Collins, 1977) were performed on virus which had been grown for two days in ten day old embryonated chicken eggs and subsequently purified by combined differential and sucrose gradient centrifugation. In all studies purified virus banded at more than one density value. Furthermore virus in each band varied in morphology as shown by negative staining electron microscopy, although the number and molecular weights of the polypeptides resolved by acrylamide gel electrophoresis were similar among virus of different densities.

There was little agreement between laboratories as to the number and size of the polypeptides resolved by PAGE (see Table 3).

this they suggest that the 125,000 molecular weight glycopeptide may be important in the haemagglutinin.

Investigations into the protein structure of the enteropathogenic bovine coronavirus (Hajer and Storz, 1979) produced results which were very different from those reported for other mammalian coronaviruses. No bands were detected in isopycnic sucrose or CsCl gradients onto which had been placed virions disrupted by Tween-80, Nonidet P40 or diethyl ether. The authors suggest that this is owing to virions being completely disassembled by these compounds. When virions were treated with chloroform and similarly processed however, core like structures 80 nm in diameter were found at a buoyant density of 1.201 g cm^{-3} in sucrose. These particles contained only non-glycosylated polypeptides of molecular weight 82,000 and 36,000.

Analyses as to the type and number of polypeptides contained in the IBV virion have come from only two laboratories in recent years (Bingham 1975, Collins et al. 1976, Alexander and Collins 1977, MacNaughton and Madge 1977, MacNaughton et al. 1977). All but one (Alexander and Collins, 1977) were performed on virus which had been grown for two days in ten day old embryonated chicken eggs and subsequently purified by combined differential and sucrose gradient centrifugation. In all studies purified virus banded at more than one density value. Furthermore virus in each band varied in morphology as shown by negative staining electron microscopy, although the number and molecular weights of the polypeptides resolved by acrylamide gel electrophoresis were similar among virus of different densities.

There was little agreement between laboratories as to the number and size of the polypeptides resolved by PAGE (see Table 3).

Bingham (1975) found two bands of virus in equilibrium sucrose gradients. As many as 16 polypeptides were found in each band, the two major peaks corresponding to molecular weights of 130,000 and 49,000. Five, of molecular weights 180,000, 130,000, 83,000, 37,000 and 14,000, were shown to be glycopeptides by Periodate-Schiff staining reactions in acrylamide gels. The carbohydrate to protein ratio ranged from 0.11 - 0.22 when protein was measured by E_{260}/E_{280} measurements. Similar results were obtained for all four strains of IBV examined: Beaudette, Massachusetts, Connecticut and H120, another strain of Massachusetts type.

The second study (Collins et al. 1976) described a much greater heterogeneity of virus bands in sucrose gradients. Five peaks, all retaining infectivity and complement fixing activity were resolved. When each peak was analysed by polyacrylamide gel electrophoresis four major polypeptides of molecular weights 52,000, 45,000, 34,000 and 32,000 were resolved. An additional ten minor peptides were also present. The proportions of polypeptides were approximately similar for all peaks: four were tentatively identified as glycoproteins of molecular weights; 94,000, 73,000, 52,000 and 32,000.

Alexander and Collins (1977) have since reported a further analysis of the conditions of purification and the number of proteins which could subsequently be resolved by PAGE in purified Beaudette strain of IBV. Sixteen polypeptides were resolved, four of which with molecular weights 86,000, 68,000, 63,5000 and 36,000, were identified as glycoproteins by Periodate-Schiff staining reactions. The method of purification was similar to that used in the earlier study, namely a combination of differential and sucrose gradient

centrifugation. In addition virus was purified from both allantoic fluid and fluid from infected chicken kidney tissue culture in which the virus had been labelled with ^3H leucine. Supplementary methods of purification including precipitation of virus with concanavalin A and Metrizamide density gradient centrifugation made no difference to the number and size of polypeptides resolved by PAGE.

The fourth study (MacNaughton and Madge, 1977) found one major and two minor virus peaks in sucrose gradients. Three IBV strains were examined and in each strain seven polypeptides (GVPI(130), VGP2(105), VP3(97), VGP4(81), VGP5(74), VP6(51) and VP7(33)) were resolved by PAGE in continuous 7.5% gels. Increasing the time of SDS sample preparation resulted in the appearance of smaller molecular weight species which the authors concluded were the result of the degradation of higher molecular weight polypeptides.

Attempts to assign individual polypeptides to their position in the IBV virion have been reported. Bingham (1975) treated intact purified IBV with bromelain and found that the projections were removed and treated virions banded at a new buoyant density of 1.15 g cm^{-3} instead of 1.18 g cm^{-3} (light form) and 1.22 g cm^{-3} (heavy form); the buoyant densities of the two bands found in sucrose gradients during purification. SDS-PAGE analysis showed that bromelain removed two of the glycopeptides, VGP130 and VGP83 and proportions of VP130, VP106 and VP70 were substantially reduced suggesting that these polypeptides are external to the membrane. Similar results were obtained for all four strains of IBV examined: Beaudette, Massachusetts, Connecticut and H120.

A further report (MacNaughton et al. 1977) described the effect of bromelain (0.7 mg/ml) and 1% nonidet P40 on purified IBV virions.

Bromelain treatment at 37°C for 15 minutes removed the surface projections of the virion as seen by electron microscopy, removing VP1, VP2 and VP5 of molecular weights 130,000, 105,000 and 74,000 respectively, concluding that these three polypeptides are components of the surface projections.

Treatment with Nonidet P40 followed by sucrose gradient centrifugation produced particles with intact coronas but with considerably reduced amounts of VP6 (molecular weight 51,000) when analysed by PAGE. These virions were easily penetrated by stain as revealed by electron microscopy. It is suggested from this evidence that VP6 is an internal component which is lost by treatment of complete virions with NP40. The polypeptides VP3, VP4 and VP7 would therefore represent virion components other than those of the projections and the RNP.

All reports agree to the presence of a major non-glycosylated polypeptide of approximately 50,000 molecular weight which is unaffected by protease treatment and therefore is likely to be inside the envelope.

Genome

Several investigators have recently confirmed that the genome of coronaviruses including IBV consists of single stranded RNA of messenger (positive) polarity together with polyadenylated tracts (Lomniczi, 1977, Lomniczi and Kennedy 1977, Schochetman et al. 1977, MacNaughton and Madge, 1977, Wege et al. 1978, Lai and Stohlman, 1978). Further studies have also indicated that the genome is present in virions as one continuous strand despite early investigations which suggested that the RNA of IBV was heterogeneous in

size ranging from 0.5 to 3.0×10^6 molecular weight (Tannock 1973). This heterogeneity in size may have resulted from the harsh conditions of extraction resulting in breakage of the RNA genome. Garwes et al. (1975) extracted single stranded RNA from both porcine coronaviruses TGEV and HEV which was characterized as consisting of a large 60-70 S component together with a minor 4S fragment. On heating above 60°C the RNA melted to give 35S fragments. From this evidence they suggest that the genome of TGEV and HEV may have a subunit structure similar to the oncogenic RNA viruses. No other coronavirus studies have confirmed this finding.

There are reports of 4S RNA being present in preparations of RNA from TGEV and HEV (Garwes et al. 1975), and IBV (Tannock, 1973, Schochetman et al. 1977). Tannock and Hierholzer (1977) also obtained a 4S component from HCV OC43 genome by heating at 37°C . Schochetman et al. (1977) showed that this portion was polyadenylated in IBV a finding contrary to that of Tannock and Hierholzer for OC43 in the RNA of which poly A tracts were detected but not associated with the 4S.

Although the genomes of different coronaviruses have the common properties mentioned above, the sizes reported for the RNA vary considerably (Table 4). Two reports on the properties of the RNA of MHV (Lai and Stohlman 1978, Wege et al. 1978) agree that it has a molecular weight of 5.4×10^6 and a sedimentation coefficient of 50-60 S. Similar values were obtained for IBV by Schochetman et al. (1977) and HCV strain OC43 by Tannock and Hierholzer (1977). However other studies have determined the molecular weight of the IBV genome to be $8-9 \times 10^6$ (Watkins et al. 1975, Lomniczi and Kennedy 1977, MacNaughton and Madge 1977).

The RNA isolated from IBV (Lomniczi 1977, Schochetman et al. 1977) and MHV (Wege et al. 1978) was found to be infectious and therefore the coronavirus genome is thought to be of messenger polarity. However it has not been demonstrated that the poly A tracts are situated towards the 5' or 3' termini of the genome, nor that capped structures are present at the 5' ends.

Replication

Early studies using immunofluorescence and electron microscopy indicated that the replication of coronaviruses appears confined to the cytoplasm (David-Ferreira and Manaker, 1965, Hamre et al. 1967, Becker et al. 1967, Berry 1967, Oshiro et al. 1971). A recent paper however, has found evidence that intranuclear viral antigens are present (2 hours post infection) in MHV infected mouse tissue culture cells. Immunofluorescence techniques were used to identify antigens of viral origin (Robb and Bond, 1979).

Electron microscopical studies on human coronaviruses growing in human diploid cells have shown that cell associated virus appears six hours after infection and reaches a maximum after 24 hours when cisternae in the endoplasmic reticulum are filled with particles. The titre of virus in tissue culture fluid reaches its maximum 36 hours post infection at which time there is only a minimal cytopathic effect. The titre of virus appears to diminish as the cell sheet degenerates (Hamre et al. 1967). In all coronaviruses the typical cytopathic effect seen in tissue culture consists of syncytia.

Electron microscopy has shown that new particles bud into cytoplasmic cisternae or vesicles, at which stage the virion obtains its outer envelope (Berry 1967, Becker et al. 1967, Hamre et al. 1967,

Oshiro et al. 1971, David-Ferreira and Manaker, 1965). The extracellular release of virus is thought to occur either by fusion of cytoplasmic vesicles containing mature particles to the plasma membrane or rupture of the vesicles as they near the surface of the cell (Hamre et al. 1967, Oshiro et al. 1971).

Tissue Culture Studies

Studies on coronaviruses have been hampered by the fastidiousness of their growth in vitro. McIntosh (1974) has reviewed the range of tissue cultures in which coronaviruses have been successfully grown. Primary epithelial cultures are derived from the species in which coronaviruses cause a natural infection. Thus FCEV and HEV may be isolated from diseased animals in primary pig kidney tissue culture (Lee, 1956, Greig et al. 1962). Similarly MHV replicates to high titre in primary mouse liver cells and mouse macrophages (Mallucci, 1965).

Coronaviruses isolated from humans and fowls do not grow as readily in cell monolayers on primary isolation from clinical specimens. Human strains similar to 229E may be successfully isolated from clinical specimens in human kidney tissue culture (Hamre and Procknow, 1966, Hamre et al. 1967). Other strains, however, may only be grown in human embryonic tracheal organ culture (Tyrrell and Bynoe, 1965, McIntosh et al. 1967a) and are prefixed by the letters OC (organ culture). There has been one report of a coronavirus from a case of human gastroenteritis being isolated in vitro. Caul and Clarke (1975) reported evidence for replication of the virus in human embryo intestinal organ cultures. However the isolate could

not be passaged. Identification of the agent as a coronavirus was solely by morphology as visualised in thin sections of organ culture cells and negatively stained preparations of the culture fluid.

Adaptation of IBV to tissue culture, where successful, has required the prior adaptation of field strains by passage in chicken embryos or chick tracheal organ cultures (Cook et al. 1976). IBV strains which have been passaged in eggs only a few times cause minimal pathological effect on the developing embryo and this increases with continued passage.

Those tissue cultures which have been shown to support the growth of IBV are primary chicken embryo kidney (Akers and Cunningham 1968), primary chicken kidney (Cunningham and Spring 1965, Churchill 1965) and Vero cells (Cunningham et al. 1972). The Beaudette strain may also be grown in BHK-21 cells but not other strains (Simpson et al. 1978). Not all strains which have been adapted to chick embryo culture may be successfully adapted to tissue culture. The highly egg adapted strain Beaudette (IBV 42) has generally been found to replicate to high titre in these cell types, producing both a plaque and a cytopathic effect. Subsequently representatives of all major antigenic types have been adapted to chicken kidney tissue culture although a variable number of blind passages appears to be necessary before a cytopathic effect is observed. In these instances the virus titre rarely rises above 10^5 EID₅₀/ml.

Organ cultures derived from chickens have been used successfully to propagate IBV. Darbyshire et al. (1976) used roller tubes

for the culture of explants from various chick tissues and studied their efficiency for the growth of IBV strains. IBV was shown to replicate in chicken tracheal organ culture in accordance with a single cycle growth curve. They found that the most susceptible tissues were nasal turbinate and tracheal tissues. There was a slight difference in tissue susceptibility to different strains which the authors suggested may have been due to differences in pathogenicity among strains.

MATERIALS AND METHODS

Virus

The Massachusetts strain (M41) of infectious bronchitis virus was studied exclusively. A virus seed was kindly supplied by Dr. Darbyshire of the Houghton Poultry Research Station and had 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 (Orchards Farm, Great Missenden, Bucks). Allantoic fluid was harvested at 2 days after each infection and fluid from the third passage used as stock virus for all experiments. Aliquots were stored frozen at -70°C until required.

Estimation of Virus Infectivity

Virus infectivity was quantitated by inoculating 0.1 ml of virus diluted in 0.05M tris-HCl buffer pH 7.5 (Sigma Chemical Co., St. Louis, Mo.) into the allantoic cavity of 10 day old embryonated hens' eggs. Embryos were examined by dissection 8 days later and virus infection scored by visible evidence of stunting, curling and membrane thickening.

Tissue Culture Methods

Chicken kidney tissue culture

Kidneys were dissected from 3 week old chickens and washed twice in phosphate buffered saline (PBS) (Oxoid, Basingstoke, Hants) containing penicillin and streptomycin. Minced tissue was passed through a sterile glass syringe to disperse the cells and then placed in PBS containing 5% trypsin. The trypsin-containing medium was renewed after 10 minutes incubation at 37°C and again after 20 minutes when it was stored at 4°C , the trypsin present having been inactivated by 1% foetal

calf serum. Twenty minutes later after dispersion of the tissue the cell suspension was filtered and any remaining trypsin inactivated with 1% foetal calf serum.

Cell suspensions were centrifuged at 700 r.p.m. in a bench centrifuge for 10 minutes at room temperature and the supernatant discarded. Cells were then resuspended in growth medium (inactivated foetal calf serum 5%, sodium bicarbonate 0.11%, penicillin and streptomycin in Basal Medium (Eagle) (BME) (Flow Laboratories, Irvine, Scotland) at a final concentration of approximately 1.1×10^6 cells per ml. Ten mls of this diluted suspension were placed in 25 cm² flasks (Falcon Plastics, Oxnand, Ca.) and incubated at 37°C. The growth medium was renewed after the first 24 hours of incubation. After five days the growth medium was decanted from the cell monolayer and 10 mls of maintenance medium added (foetal calf serum 5%, sodium bicarbonate 0.11%, penicillin and streptomycin in BME).

Chicken embryo kidney tissue culture

Kidneys were obtained from 20 day old chicken embryos by the method of Cunningham (1963). Eggs were washed with aqueous ethanol and the shell above the air sac removed with sterile forceps. The embryos were then removed aseptically from the shell and the egg sac removed and discarded. The embryos were dissected and the kidneys removed and placed in sterile PBS. The preparation of monolayers from this stage was similar to that for chicken kidneys, confluence being visible within 2 days of preparation.

Virus inoculation of chicken kidney tissue culture.

C.P.E.

Cell sheets were washed thoroughly with the BME containing 0.11% sodium bicarbonate with added penicillin and streptomycin and drained. Virus was diluted in the above medium and 0.2 ml aliquots were placed on the monolayers and left to absorb at room temperature or at 37°C for 2 hours. Ten mls of maintenance medium were then added to the cells and the flasks incubated at 35°C, and maintenance medium was changed every 3 days.

Plaque Assay

Chicken kidney cells were washed thoroughly with 199 medium (Flow Laboratories) containing 0.11% sodium bicarbonate, penicillin and streptomycin and drained. Virus was diluted in the same medium and 0.2 ml aliquots were placed on the monolayers and left to absorb at room temperature or at 37°C for 2 hours. The monolayers were overlaid with 199 medium containing iron agar 0.75%, 199, sodium bicarbonate 0.11%, inactivated foetal calf serum 1.0%, penicillin and streptomycin and incubated at 35°C. Cells were stained with 4 mls of 0.1% neutral red in PBS.

Preparation of Antisera

Antisera to IBV were raised in guinea pigs (Hartley strain, male, 8 months, 1250 gm). The immunising antigen was purified virus, 10-25 µg total protein, combined 1:1 with Freund's Complete Adjuvant (Difco, Detroit, Michigan). The animals were bled prior to immunisation and then two preparations of antigen were inoculated intramuscularly one month apart into alternate thighs. The animals were bled 10 days after the booster dose, defibrinated with Alsever's solution, the sera separated, and stored at -70°C until required.

Complement Fixation Tests

The method used was adapted from that detailed by Grist et al. (1974). All tests were carried out in microtitre plates (Sterilin Products, Teddington, England) and fixation of complement was overnight at 4°C.

Immunodiffusion

6 cm x 3 cm microscope slides were washed in 10% HCl in ethanol and 0.1% agar film applied. Treated slides were then overlaid to a thickness of approximately 1 mm with 0.9% agarose in PBS. After cooling wells 2 mm in diameter and 3 mm apart were cut using a template. Approximately 50 µl of sample was applied to each well and the slides developed at room temperature in a moist atmosphere.

Radioimmunoprecipitation

¹²⁵I-IBV was diluted in PBS containing 0.25% gelatin to approximately 3,000 c.p.m. per ml and dispensed in aliquots of 0.5 ml. Guinea pig anti-IBV sera was added to a final concentration of 10%, mixed and incubated at 37°C for one hour and overnight at 4°C. The next day rabbit anti-guinea pig IgG sera (Miles Ltd, Elkhart, Indiana) was added to a final concentration of 1/25, mixed well and the reactants left at 4°C overnight. After incubation the precipitates were pelleted at 3,000 on a bench centrifuge for 15 minutes. The supernatant was removed and the distribution of radioactivity determined by counting both pellet and supernatants in a gamma spectrometer.

Haemagglutination

Red blood cells were obtained from 3 week old chickens, collected into heparin (1 IU/ml), washed three times in PBS then stored at a

concentration of 10% in dextrose gelatin veronal buffer. The concentration of the red cells was adjusted to 0.05% in PBS on day of use.

Virus was obtained from infected allantoic fluid which had been harvested after 40 hours incubation at 37°C and clarified at 10,000 g. As indicated below virus was either used unconcentrated or concentrated 100 times by centrifuging the allantoic fluid at 40,000 g for one hour in a Beckman SW40 rotor at 4°C and the pellet resuspended in PBS.

The negative control consisted of uninfected allantoic fluid treated similarly to infected fluid.

Phospholipase C' Treatment of Virus

Virus was treated with phospholipase C' according to the method of Bingham et al, (1975).

Equal volumes of virus and phospholipase C' (Lecithinase C, EC 3.1.4.3., type 1 from Clostridium welchii, Sigma) diluted to 1 IU/ml were preheated individually at 37°C for 10 minutes. They were then added together, mixed thoroughly by vortex mixer and incubated at 37°C for 30 minutes. After heating the mixtures were cooled immediately in an ice bath and tested for haemagglutination (HA) activity.

Haemagglutination Titration

PBS buffer was used throughout, and all reagents and reactions were at 4°C unless otherwise specified. Samples to be tested for HA were diluted 2-fold, in microtitre plates with U-shaped wells (Sterilin Products) placed on iced water and equal volumes of 0.5% chick red blood cells added.

Effect of divalent cations on HA

Stock solutions of CaCl_2 2%, and MgCl_2 2% in distilled water were prepared and diluted 1/200 for use.

Virus Purification

Approximately 100 EID_{50} of virus stock were inoculated into 10 day old embryonated hens' eggs and incubated for 40 hours at 37°C . The eggs were chilled for a minimum of 4 hours at 4°C and the allantoic fluid harvested. Virus purification was begun without delay after the removal of cellular debris by centrifugation for 30 minutes at 10,000g. Where possible all steps were performed at 4°C . Sodium chloride was added to the allantoic fluid with constant stirring to a final concentration of 0.46 M. A 50% solution of polyethylene glycol 6000 (PEG) (Union Carbide, U.K.) in distilled water was then added dropwise until the final concentration reached 8.7% w/v. The mixture was left for a further 12 hours at 4°C , centrifuged for 30 minutes at 10,000 g and the pellet resuspended in 0.02 M tris-HCl buffer pH 7.0 containing 0.2M glycine and 0.002M EDTA (GNTE buffer). The final volume corresponded to 1/200th of the original volume of allantoic fluid. Resuspended virus was sonicated in a bath type sonicator (Headland 3 transducer activated 150 watt) for 1 minute and then centrifuged at 10,000 g for ten minutes.

The concentrated virus suspension was incorporated into the top 5 mls of a 5-45% w/v Metrizamide (Nyegaard and Co. AS.Oslo) gradient in GNTE buffer and centrifuged at 50,000 g in a Beckman SW40 rotor at 4°C for 8 hours. The band containing the peak of infectivity was recovered and separated from Metrizamide by chromatography through a 1.5 cm x 20 cm column of Sephadex G-75 (Pharmacia Fine Chemicals,

Uppsala) equilibrated with GNTe buffer. The equilibrium centrifugation step was repeated and the purified virus again separated from Metrizamide by chromatography through a 0.9 cm x 10 cm column of Sephadex G-75. Virus was either immediately tracers labelled or stored at -70°C prior to analysis.

Electron Microscopy

Samples for electron microscopy were placed onto Formvar/carbon 400 copper grids (Smethurst No. 400: Smethurst Highlight Ltd. England), washed by inverting onto the surface of distilled water in a container, and stained with 2% phosphotungstic acid adjusted to pH 6.0 with KOH. The grids were examined using an AEI 801 electron microscope at an operating voltage of 80 Kv.

Electrophoretic Procedures

Polypeptide analysis of solubilised IBV was performed by the electrophoretic separation in polyacrylamide gels using essentially a high pH/discontinuous buffer system (Laemmli, 1970, Maizel, 1971).

Gels were prepared in 5 mm diameter precision glass tubes to a final length of 10 cm. The desired concentration of polyacrylamide was obtained by dilution of a stock solution containing 28.6% w/v acrylamide and 1.4% w/v methylene bisacrylamide (Eastman Kodak Ltd, Rochester, N.Y.) in distilled water. Tris-HCl buffer pH 8.7 was added to a final molarity of 0.375 M. Polymerization was effected by the addition of $\text{N}_1\text{N}_1\text{N}_1\text{N}_1$ tetramethyl-ethylenediamine and ammonium persulphate to final concentrations of 0.04% and 0.2% respectively. After polymerization, the resolving gels were overlaid with 1 cm stacking gel containing 4% w/v acrylamide and buffered with 0.06 M tris-phosphate buffer pH 6.7.

In some experiments, gradient slab gels were prepared essentially as described by Marsden et al, (1976). A linear gradient of polyacrylamide concentration was formed between two 14 cm x 14 cm glass plates held 1.5 mm apart by perspex spacers. Starting and final concentrations were 15% and 5% respectively. Slots for sample application were formed in the stacking gel prepared to a final depth of 2 cm over the resolving gel. In both systems 0.005 M tris-HCl 0.033 M glycine buffer containing 0.1% sodium dodecyl sulphate (SDS) (Sigma Chemical Co.) was used for both anode and cathode buffer reservoirs.

Samples for electrophoretic analysis were solubilised in buffer containing 0.06 M tris-phosphate buffer pH 6.7 and 2% SDS. When required 2-mercaptoethanol (B.D.H. Chemicals Ltd, Poole) was added to a final concentration of 5% v/v. Disruption was by heating at temperatures specified in Results. Electrophoretic separation was performed at a constant current with a maximum of 2.0 mA/gel for cylindrical gels or 8mA for slab gels. Bromophenol Blue was added to the samples as a tracking dye.

After electrophoresis gels were fixed in solution containing methanol:acetic acid:water (64: 23: 64). Components were visualised by staining with 0.25% Coomassie Brilliant Blue R grade (Charles A. Lamb Ltd, North Acton, London) dissolved in fixative solution. After two hours at room temperature, gels were destained in 5% methanol and 7.5% acetic acid. Reference proteins were run in parallel gels as markers of molecular weights and included phosphorylase a (molecular weight 93,000) bovine serum albumin (molecular weight 69,000) ovalbumin (molecular weight 43,300) chymotrypsinogen A (molecular weight 25,500), and lysozyme (molecular weight 14,300) (Sigma Chemical Co.). 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.

Radiolabelling Procedures

Purified IBV was radiolabelled by reaction with an iodinated acylating agent. Approximately 5 µg of purified virus was added to 0.3 ml (500 µCi) of N-succinimidyl 3-(4-hydroxy, 5-¹²⁵I) iodophenyl) propionate (Bolton and Hunter, 1973). The iodinated ester was purchased from The Radiochemical Centre Ltd, Amersham (Product No. 1MS.861) as a suspension in benzene. The required amount of reagent was transferred to the reaction vessel and the solvent evaporated under nitrogen before the addition of protein. Acylation was for 5 hours at 4°C in 50 µl of 0.2M borate buffer pH 8.5. Approximately 500 µl of 0.2 M glycine in borate buffer was then added to inactivate any remaining iodinated ester and after 1 hour at 4°C the labelled virus was separated from other reaction products by gel filtration through a prepacked column of Sephadex G-25 (Product No. PD10, Pharmacia Fine Chemicals) equilibrated in PBS containing 0.25% gelatin. Radiolabelled virus prepared in this manner was stored for up to 1 week at 4°C or at -70°C for up to 1 month prior to further analysis.

Chemical Analyses

Estimation of Total Protein Content

The gross amount of protein was estimated by a modification of the technique originally published by Lowry et al. (1951). To 200 µl of sample was sequentially added 1 ml of 0.01% copper sulphate and 0.02% potassium tartrate in 0.1 M NaOH containing 2% sodium carbonate. After 10 minutes at room temperature 100 µl of Folin-reagent (B.D.H.

Chemicals Ltd) diluted 1:1 in distilled water was added by vortex mixing and the development of a blue colour allowed to proceed for 30 minutes prior to the measurement of absorbance at 660 nm. Protein concentration was estimated with reference to a curve obtained with crystalline bovine serum albumin (Sigma Chemicals Ltd.) dissolved to predetermined concentration.

The presence of glycinate ions in samples for protein estimation decreased by approximately 50% the absorbance values obtained by the Lowry procedure. Therefore an alternative method for protein estimation which did not involve the reaction of reagent with $-NH_2$ groups (Schaffner et al., 1973) was used for samples in GNTS buffer.

Samples containing 0.5-30 μ g protein were placed in 8 x 60 mm test tubes and water added to a final volume of 0.27 ml. To this were added 0.03 ml 1M tris-HCl buffer pH 7.5 containing 1% sodium dodecyl sulphate followed by 0.06 ml of 60% trichloroacetic acid (TCA). The sample was then mixed on a vortex mixer and left at room temperature for more than 2 minutes. The mixture was taken up into a pasteur pipette and spotted onto a Millipore filter (HAWP 025C0 45) under suction, taking care that the area used was no larger than 7 mm in diameter. The tube was rinsed with 0.3 ml 6% TCA and filtered through the same spot. Up to 3 samples could be placed on each membrane and the whole area rinsed with 4 ml 6% TCA.

The membrane filter was stained for 2-3 minutes in 0.1% amido Schwarz 12B in methanol:glacial acetic acid:distilled water (45: 10: 45), rinsed for 30 seconds in distilled water and then rinsed again for 1 minute, 3 times using 200 ml volumes of destaining solution (methanol:glacial acetic acid:distilled water, 90: 2: 8). The destaining solution was removed by placing the filter in distilled

water for 2 minutes. The membranes were blotted dry and the blue dot removed with a cork borer. Each disc was then transferred to a small test tube or plastic bijoux and 0.6 ml eluant (25mM NaOH, 0.05mM EDTA in 50% aqueous ethanol) was added. After 10 minutes during which time the tube was mixed 3 times on a vortex mixer, the absorbance was read at 630nm.

The Schaffner method for the estimation of protein can detect as little as 0.5 μ g of protein. This degree of sensitivity compares favourably with that of the Lowry method which can detect 0.2 μ g.

¹²⁵Iodinated Concanavalin A Labelling of IBV Polypeptides

100 μ g of concanavalin A (Pharmacia Fine Chemicals) was radio-labelled by the addition of Bolton and Hunter reagent according to the method used for labelling purified IBV (see above) and stored at -70°C prior to use. Protein components of purified IBV were separated in polyacrylamide gels by electrophoresis, fixed and stained. The gels were washed for at least two days in several changes of 0.05M tris-HCl buffer pH 7.2 containing 0.14 NaCl and then cut into 1 mm slices. Each slice was immersed in 100 μ l of 0.05 M tris-HCl buffer pH 7.2 containing 0.14 M NaCl, 1mM CaCl_2 , 1mM MnCl_2 , 0.5% haemaglobin and ¹²⁵I-concanavalin A (diluted to approximately 1×10^5 cpm per gel slice). A control gel was similarly treated except that Mn^{++} and Ca^{++} were not added to the buffer and 5% methyl mannoside (Sigma Chemical Co.) was included. The slices were incubated in buffer overnight at room temperature and then washed 3 times in 0.05M tris buffer. The presence of residual radioactivity was then determined for each slice.

Treatment of Virus with Bromelain

Bromelain was obtained in powdered form from Serva Feinbiochemica, Heidelberg. Purified virus was heated in GNT buffer containing 0.01, 0.05 and 0.1% bromelain for 1.5 hours at 37°C. Treated virus was then placed directly onto a 10-40% w/v Metrizamide gradient in GNT buffer and centrifuged for 10 hours at 50,000 g in a Sorvall AH-650 rotor. A control preparation was similarly heated in the absence of the enzyme and centrifuged in parallel with treated virus. Bromelain treated and control viruses were then dialysed overnight against GNT buffer at 4°C and analysed by both electron microscopy and SDS-PAGE.

Triton X-100 Disruption of Virus

Con A Chromatography

Triton X-100 (scintillation grade) was obtained from B.D.H. Chemicals Ltd, Poole. Radiolabelled virus was solubilised in 2% Triton X-100 in GNT buffer containing 0.5% NaCl for 4 hours at 37°C. Treated virus was then placed directly onto a 0.9 x 10 cm column packed with 6 mls bed volume of Con A-Sepharose (Pharmacia Fine Chemicals) equilibrated in 0.1M tris-HCl buffer pH 7.3 containing 0.5M NaCl, 2% Triton X-100 1mM CaCl₂, 1mM MnCl₂. The eluant from the column was collected in 0.5 ml fractions and the radioactivity in them determined. After 30 mls had been collected from the column the eluant buffer was changed to 0.1M tris-HCl pH 7.3 containing 0.5M NaCl, 2% Triton X-100 and 5% methyl mannoside. Fractions were again collected and the radioactivity in them determined.

Fractions containing the radioactivity 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 hours at 37°C. Treated virus was then placed directly on to a 20-65% w/w sucrose gradient in GNTe buffer and centrifuged for 24 hours at 150,000 g in a Sorval AH-650 rotor. Protein bands were dialysed against GNTe buffer for 6 hours at 4°C then analysed by electron microscopy and SDS-PAGE.

The experiment was repeated using Metrizamide as the gradient material.

RESULTS

Virus Growth in Tissue Culture

Attempts to adapt the Massachusetts strain of IBV to primary chicken kidney cell cultures were unsuccessful, even after four blind passages. Neither a cytopathic effect nor the appearance of viral plaques were seen regardless of the type of overlay employed. The addition of trypsin to the overlay had no effect. Therefore the production of virus for biochemical studies and assays for infectivity were performed in embryonated chicken eggs.

Serological Techniques

Immunodiffusion

No precipitin lines were detected in agarose gel diffusion tests by antisera produced in guinea pigs in response to intramuscular inoculation of purified IBV. Antigens included neat and concentrated allantoic fluid (containing 10^8 EID₅₀/ml and 10^{10} EID₅₀/ml of infectious virus respectively). In addition precipitin lines were not detected when purified virus disrupted with Triton X-100 was used as an antigen.

Complement Fixation

Guinea pig antisera were found to crossreact to high titre (1/128) with uninfected allantoic fluid. The peak of complement fixing activity did not correspond to the peak of virus infectivity after isopycnic centrifugation in linear gradients of Metrizamide (Fig. 2).

Neutralisation

Using a constant virus dose (100 EID₅₀/ml) and varying the antibody concentration a neutralisation titre of 1:120 was obtained with infectivity in embryonated eggs as the indicator system.

Owing to the relatively poor reactivity with purified IBV and the high degree of cross reactivity with normal allantoic fluid, guinea pig antisera were not used further for the characterization of IBV.

Haemagglutination

Concentrated suspensions of IBV did not haemagglutinate chicken or guinea pig red cells unless the virus was first treated with phospholipase C'. Varying the pH of the reaction of both phospholipase treatment and haemagglutination influenced the resulting HA titre, optimum results being obtained at pH 6.4. The addition of divalent cations slightly raised the titre and resulted in a pattern more easily read by eye. Haemagglutination did not occur with allantoic fluid in the absence of virus.

The effect of diluting suspensions of virus prior to phospholipase C' treatment was investigated and it was found that the titres obtained for virus diluted before treatment did not correspond with those for virus treated with the enzyme and then diluted. These results indicated that haemagglutination by IBV obtained after enzyme treatment could not be used to quantitate virus and therefore HA was not used as a particle assay for virus during purification and analysis.

Virus Purification

PEG Precipitation

The precipitation of virus from infected allantoic fluid was achieved by the addition of PEG 6000 and resulted in a 200-fold concentration of virus with little or no significant loss of infectivity (Table 5). Optimum results were obtained by adding PEG to clarified allantoic fluid immediately after harvesting from infected eggs. The concentration of NaCl before addition of PEG was found to be critical. In the absence of NaCl a loose flocculent precipitate was obtained that was found difficult to collect by centrifugation and there was a concomitant loss of infectivity. Addition of NaCl in excess of the optimum concentration of 0.46M before addition of PEG resulted in a heavy granular precipitate that could not be resuspended after centrifugation. The recovery of infectivity was also considerably reduced under these conditions.

Gel Chromatography

Attempts were made to remove residual PEG and small molecular weight compounds from the PEG pellet by gel chromatography. The properties of two gel media were investigated. The resuspended virus pellet was fractionated by column chromatography in Bio-gel A-5m agarose (Bio-Rad Laboratories). Virus was present in the void volume of the column whereas PEG was eluted much later (Fig. 3). However, as the recovery of the infectious virus dropped 10-fold this system was excluded as a suitable purification step. Column chromatography through Sephadex G-75 also removed PEG from the virus (Fig. 4) without loss of infectivity. However the gel was rapidly clogged by PEG and each bed could be used for only one sample application.

As a result the decision was made not to include gel chromatography as a purification step immediately after precipitation with polyethylene glycol.

Centrifugation

Further concentration and purification was then carried out by two successive equilibrium centrifugations in 5-45% Metrizamide gradients. Although up to 5 visible bands were present after the first centrifugation step in Metrizamide, infectivity was restricted to a single band with an equivalent buoyant density of 1.14 g cm^{-3} . Further equilibrium centrifugation of this material produced a single band of the same buoyant density which contained all recovered virus infectivity. Examination by electron microscopy of the final preparation showed the presence of virus particles with a morphology consistent with that of the coronaviruses (Fig. 1). The majority of particles had retained the distinctive fringe of outer projections. During the course of these studies, it was noticed that the use of centrifugal forces in excess of 100,000 g markedly reduced the number of intact virus particles. This was paralleled by a reduction in the amount of infectious virus recovered.

Separation of the virus from Metrizamide after each centrifugation run was accomplished by column chromatography through Sephadex G-75. This method was rapid and efficient and thus was preferred to removal of Metrizamide by dialysis.

Total recoveries of protein and infectivity at each stage are shown in Table 5.

Similar recoveries of protein and infectivity were achieved when parallel purifications were run using sucrose as the gradient material instead of Metrizamide. However isopycnic banding of the virus in Metrizamide required only 7 hours and prolonged centrifugation did not alter the buoyant density value of 1.14 g cm^{-3} . In contrast the peak of virus infectivity in sucrose gradients had reached a density of only 1.16 g cm^{-3} after this interval and centrifugation for another 5 hours was necessary for the virus to reach its buoyant density of 1.18 g cm^{-3} in sucrose. Electron micrographs of purified virus prepared in sucrose showed that virions were penetrated by stain and often bulges were present in the envelope (see Fig. 1b) unlike particles prepared in Metrizamide.

Figure 5 shows the SDS-PAGE profiles of virus purified in Metrizamide and sucrose after two consecutive isopycnic bandings. With both media two centrifugation steps were necessary to remove the very high and very low molecular weight material.

Radiolabelling of purified IBV

Virus purified by combined PEG precipitation and equilibrium centrifugation in Metrizamide was radioiodinated as described in Materials and Methods. Two methods of radioiodination were investigated: a modification of the chloramine-T procedure as described by Hunter and Greenwood (1962) and the method of Bolton and Hunter (1973).

Chloramine-T Method

Oxidation of carrier free Na^{125}I in the presence of the oxidizing agent chloramine-T released free ^{125}I ions which were incorporated in the protein of virus present in the reaction mixture. The reactants

were eluted on a Sephadex G-75 column and the radioactivity in the void volume measured (see Fig. 6). Only 32% of radioactivity was precipitated by 10% trichloroacetic acid. The addition of 25 µg chloramine-T per µg purified IBV in the presence of 500 µCi Na¹²⁵I gave a specific activity of 32 µCi/µg.

When the properties of the radiolabelled virus were further analysed by equilibrium centrifugation in 5-45% w/v urographin gradients the peak of radioactivity was found at the top of the gradient indicating that radioiodination of IBV under these conditions destroyed the integrity of the virus particle.

Method of Bolton and Hunter

Radioiodination of IBV particles by the technique described by Bolton and Hunter (1973) (see Materials and Methods section) resulted in between 9 and 20% of the radioactivity present in the reaction mixture being routinely introduced into the purified virus particles, giving an approximate specific activity of 6 µCi/µg protein.

When reactants from the radiolabeling procedure were eluted on a Sephadex G-25 column the radioactivity in the void volume was found to precipitate in 10% TCA to above 95%. This value decreased markedly after storage at 4°C for longer than one week.

Incubation of radiolabelled IBV in hyperimmune guinea pig anti-IBV diluted 1:10 resulted in 70 - 80% of the radioactivity being precipitated after the addition of anti-guinea pig serum.

Isopycnic centrifugation of the radiolabelled product in Metrizamide showed that the radioactivity was present as a single homogeneous band at a buoyant density of 1.12 g cm⁻³ (Fig. 7). This

value was lower than that for the untreated purified virus control which banded at 1.14 g cm^{-3} . The labelling procedure did not therefore, disrupt the virus although there was a slight change in its sedimentation properties.

SDS-PAGE ANALYSIS of IBV

A total of three major polypeptide species were resolved by SDS-PAGE analysis of purified virus (Fig. 8a). After staining with Coomassie Brilliant Blue the major components were estimated to possess molecular weights of 90,000, 52,000 and 29,000 respectively. In addition, two minor components with molecular weights of 38,000 and 48,000 were consistently present, and a further two minor components with molecular weights of 70,000 and 60,000 were occasionally resolved. When gradient slab gels of 5-15% polyacrylamide were used for PAGE analysis of IBV neither high nor low components were resolved (Fig. 5) and the polypeptide profiles were similar to those obtained on cylindrical gels.

Effect of Gel Concentration

The effect of varying the polyacrylamide gel concentration on PAGE profiles was investigated. Three concentrations of gel were used in cylindrical gels; 7.5% (0.35% bis-acrylamide), 10% (0.47% bis-acrylamide), 10% (0.27% bis-acrylamide) and 12.5% (0.27% bis-acrylamide).

Similar polypeptide profiles were obtained at all concentrations except at 7.5% when the minor polypeptides at 38,000 and 48,000 molecular weights were inadequately resolved (Fig. 9a).

Effect of Reducing Agents and Temperature

The effect of solubilising virus in the presence and absence of 2-mercaptoethanol was examined.

When virus was solubilised in the absence of the reducing agent the amount of the 29,000 polypeptide present was considerably reduced (see Fig. 8b) suggesting that disulphide bridges may be important in the structure of IBV virions.

Virus radiolabelled by the Bolton and Hunter method was also analysed by SDS-PAGE. The resulting peaks of radioactivity corresponded to polypeptides with molecular weights of 29,000, 52,000 and 90,000 with minor peaks of 38,000 and 46,000 thereby indicating that all polypeptide species were radiolabelled (Fig. 10a).

The effect of solubilising virus at different temperatures both in the presence and absence of 2-mercaptoethanol was examined. Solubilisation of labelled virus in the presence of the reducing agent at room temperature for 3 hours, 37°C for 2 hours, 60°C for 1 hour or at 80°C for 10 minutes produced little change in either the number or the estimated molecular weights of the resolved polypeptides as compared to heating at 100°C for 2 minutes (Fig. 10). However, solubilisation at all three temperatures in the absence of 2-mercaptoethanol reduced the amount of the 29,000 molecular weight polypeptides present (Fig. 11).

A similar experiment was performed with unlabelled purified virus. Again there was no change in the number of polypeptides resolved after preparation of the samples at different temperatures. However in the absence of reducing agent the amount of the 29,000 molecular weight polypeptide was considerably reduced similar to the result obtained with labelled virus; in addition the 90,000 molecular

weight species ran at a slightly higher mobility (see Fig. 12) suggesting that this polypeptide may contain intrapeptide disulphide bridges. The effect of 2-mercaptoethanol on the PAGE profiles may be seen in figure 12. The slots 3-7 of the slab gel contain virus prepared at different temperatures in the absence of 2-mercaptoethanol. Slots on either side contain 2-mercaptoethanol and this has diffused across to affect the number and mobilities of polypeptides resolved in neighbouring areas of the gel.

The identification of IBV glycoproteins

Polyacrylamide gels containing reduced or unreduced polypeptide species of solubilised, unlabelled virus were incubated with ^{125}I -concanavalin A. After extensive washing, the counting of sliced gels containing reduced protein revealed the reaction of the lectin with two polypeptide species, corresponding to the major components with estimated molecular weights of 90,000 and 29,000 (Fig. 13). No reaction occurred in the presence of 5% methyl mannoside suggesting that these reactions were specific for carbohydrate. Components separated in the absence of 2-mercaptoethanol were similarly examined. Addition of ^{125}I -concanavalin A resulted in the resolution of a third glycosylated component with an estimated molecular weight of 50,000 (Fig. 13). Also present was a peak at 26,000 which did not stain with Coomassie Blue, suggesting that the 26,000 molecular weight polypeptide is highly glycosylated.

Effect of bromelain on IBV polypeptides

When virus treated with 0.05% and 0.1% bromelain was subjected to equilibrium centrifugation in Metrizamide a single band of protein

was recovered for both concentrations at a buoyant density of 1.14 g cm^{-3} . This was similar to the untreated virus control. Treatment of intact virus particles with 0.05 U bromelain resulted in no apparent change in morphology as visualised by electron microscopy (Fig. 14a). However, raising the concentration of bromelain to 0.1 U resulted in the removal of projections (Fig. 14b).

Analysis of the protein bands by SDS-PAGE resulted in the appearance of several new polypeptides and a corresponding reduction in the amounts of all native virion proteins (Fig. 15). From these results alone no conclusion could be reached as to the position in the virion of any protein component.

Further evidence of the importance of disulphide bonds to the structure of IPV was derived from studies involving the binding of ^{125}I -concanavalin A to polyacrylamide gels of bromelain treated virus. In the absence of 2-mercaptoethanol the 90,000 molecular weight polypeptide was resolved by PAGE of virus that had been treated with 0.05 U bromelain (Fig. 16b) but was absent when PAGE was performed under reducing conditions (Fig. 16a). This suggests that the protein was not completely digested but was cleaved at certain points by the protease and held intact by disulphide bridges. These bridges were reduced by 2-mercaptoethanol and the smaller species thus produced migrated separately at higher mobilities. When glycoproteins in gels of bromelain treated virus were labelled with ^{125}I -concanavalin A, at least two new glycoproteins were resolved (Fig. 17). Those corresponding to molecular weights of 60,000 and 40,000 were most likely to be breakdown products of the 90,000 molecular weight peptide. Under non-reducing conditions these glycoproteins were not resolved (Fig. 17).

Triton X-100 Disruption of IDV

Attempts were made to separate virion components by solubilising radiolabelled virus in 2% Triton X-100 for two hours at 37°C and applying the products to a Con A Sepharose affinity chromatography column. Figure 18 shows the elution profile of radioactivity after Triton disruption. A major portion of the radioactivity was present in the void volume (67%) and only 2% was eluted after application of 5% methyl mannoside. A large proportion (31%) of radioactivity was not recovered from the column. Both peaks of radioactivity were more than 95% precipitable in 10% TCA.

The peaks from the affinity column were analysed by PAGE after dialysis to remove methyl mannoside and treatment with gelatinised Bio-beads to remove the detergent (Holloway, 1973). In early experiments no peaks of radioactivity were obtained in SDS gels of void volume fractions. However if the first fractions eluted in the void volume were analysed separately from later fractions, peaks could be resolved by PAGE. Thus when fractions 3 and 9 were run on 10% polyacrylamide gels after solubilisation in 2% SDS and 5% 2-mercaptoethanol a peak of radioactivity was obtained corresponding to a molecular weight of 52,000 with minor peaks at 42,000, 35,000 and 15,000 (Fig. 19). The minor peaks did not correspond to any polypeptide found in whole virus. No peaks were resolved in polyacrylamide gels of the later fractions in the void volume even though they contained the bulk of the radioactivity.

When fractions eluted from the Con-A column after application of methyl mannoside were analysed by PAGE two peaks of radioactivity were obtained (Fig. 20). One of these corresponded to a molecular weight of 30,000 and the molecular weight of the other varied between

50,000-60,000. In no fractions was there resolved a peak corresponding to the 90,000 molecular weight glycoprotein present in whole virus. Thirty-one % of the total radioactivity applied to the column was not eluted and this may be in part associated with the 90,000 molecular weight glycoprotein which may have become irreversibly bound to the Con A Sepharose.

These results suggest that a 50,000 molecular weight polypeptide may be separated from the other virion components by solubilisation of whole virus in Triton X-100 and that it is not glycosylated since it does not bind to Con A. However a further polypeptide in this size range was eluted from the Con-A after washing with methyl mannoside. Labelling of peptides resolved in polyacrylamide gels with 125 I-concanavalin A (see above) indicated that there was not a glycopeptide in the 50,000 molecular weight region under reducing conditions. Possibly this second 50,000 species was associated in some way with the 30,000 molecular weight peptide which was also eluted after methyl mannoside washing of the column and which has been shown to be glycosylated.

Since a proportion of the radioactivity applied to the column was not eluted and a major proportion of that which was eluted could not be analysed by SDS-PAGE, other techniques for the separation of virion components were examined.

Triton X-100 disruption of virus

Separation by Isopycnic Centrifugation

Virus was solubilised in 2% Triton X-100 and its component proteins separated by isopycnic centrifugation in linear sucrose gradients (Fig. 21). Two bands containing protein were resolved.

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The buoyant density of the lighter band was 1.23 g cm^{-3} . The other band was in the bottom fraction of the gradient at a density of 1.32 g cm^{-3} . SDS-PAGE of the lighter band resolved three polypeptides of molecular weight 90,000, 52,000 and 29,000 (Fig. 22). ^{125}I -concanavalin A labelling of the gel indicated that the peptides of 90,000 and 29,000 molecular weight were glycosylated (Fig. 23). Electron micrographs 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. Resolution was not fine enough to distinguish differences between particles (Fig. 24).

SDS-PAGE of the material which pelleted to the bottom of the gradient resolved a non-glycosylated polypeptide of molecular weight 52,000 (Fig. 25) and a glycoprotein of 26,000 molecular weight which could only be detected by ^{125}I -concanavalin A labelling of the gels. (Fig. 26). The A254/A280 absorbance ratio of this band was approximately 2 compared with 1 for the band at a density of 1.23 g cm^{-3} , suggesting the presence of RNA in the band with the heavier buoyant density. Electron micrographs showed this material contained amorphous particles

When the experiment was repeated and isopycnic centrifugation was performed in a linear 10-50% Metrizamide gradient two bands of protein were again recovered (Fig. 27). The lighter band with a buoyant density of 1.22 g cm^{-3} contained particles similar in appearance to the particles banding at a buoyant density of 1.23 g cm^{-3} in sucrose (Fig. 28). The denser subviral particles pelleted through a density of 1.32 g cm^{-3} in Metrizamide and were visualised as aggregates of spheres 25-45 nm in diameter by electron microscopy (Fig. 29). The SDS-PAGE profiles of both types of particles were identical to the corresponding particles separated in sucrose.

SDS-PAGE of both types of particles was performed with and without 2-mercaptoethanol and gels were labelled with ^{125}I -concanavalin A. Two glycoproteins of molecular weight 90,000 and 29,000 were resolved in the light band containing the spikes when gels were run in reduced conditions (Fig. 23a). When 2-mercaptoethanol was not present, however, the 29,000 molecular weight peak was missing as was the 26,000 peak which was always resolved in intact virus under the same conditions (Fig. 23b). When gels of the dense spherical particle were similarly analysed by ^{125}I -concanavalin A labelling the low molecular weight glycopeptide 26,000 was resolved whether 2-mercaptoethanol was present or not (Fig. 26). Figure 26 shows that there were traces of a 90,000 molecular weight polypeptide associated with the dense particle even though this band did not stain with Coomassie Brilliant Blue.

Similar experiments were performed using purified IBV radio-labelled by Bolton and Hunter reagent. ^{125}I -IBV solubilised in Triton X-100 was then placed onto a 20-60% w/v Metrizamide gradient. The distribution of radioactivity in the gradient after isopycnic centrifugation may be seen in Fig. 27. There were two peaks of radioactivity; one near the top of the gradient which was probably associated with lipid only and the other at a density of 1.17 g cm^{-3} which corresponds to the buoyant density of the spike shaped material produced when unlabelled virus is similarly treated with Triton X-100. There was no radioactivity at the bottom of the gradient indicating that the core material had not be radiolabelled by Bolton and Hunter reagent.

SDS-PAGE of the peaks of radioactivity showed that no polypeptides were associated with the material at the top of the gradient. The band at a density of 1.17 g cm^{-3} contained peaks corresponding to molecular weights of 90,000, 52,000 and 29,000 (Fig. 30) which values are similar to those for unlabelled virus polypeptides contained in the same band.

DISCUSSION

Several comparative studies have investigated the structure and chemical composition of coronaviruses. Most studies of mammalian coronaviruses have indicated the presence of four to seven polypeptides with some variation as to the size of the observed major components (Garwes and Pocock, 1975, Hierholzer, 1976, Sturman, 1977, Wege et al., 1979 Table II). In contrast, analyses of infectious bronchitis virus (IBV) have given extremely variable results. For example, Bingham (1975) found a total of 16 polypeptides whereas MacNaughton and Madge (1977) in a more recent study consistently resolved only seven. This reported variation may reflect the widely different procedures adopted by different laboratories for both purification and analysis.

Although initial studies of IBV were confined to the Beaudette strain (IBV-42) several strains have been subsequently analysed including the Massachusetts (IBV-41) and Connecticut (IBV-46) strains. Although serologically similar to the Massachusetts strain, the Beaudette strain has been subjected to high passage in eggs with a resulting loss of infectivity for chickens (McIntosh, 1974). In addition there is some evidence that the Beaudette strain contains a corona of fewer projections than the other strains (Harkness and Bracewell, 1974). However, this observation has not been directly correlated with changes in pathogenicity. The Massachusetts strain has been found to be the most effective vaccine strain (Winterfield, 1968), inducing antibody which gives higher protection against challenge by heterologous serotypes of IBV (Hofstad, 1961). In the present study, the Massachusetts strain was used throughout as a model of IBV.

A. Purification

The Massachusetts isolate obtained from Houghton Poultry Research Station for use in these studies was passaged and routinely grown in embryonated hens' eggs. Repeated attempts to grow this strain of IBV in chick kidney cell cultures were unsuccessful. As a result it was not possible to monitor biological activity by the development of either plaques or cytopathic effect in monolayers, and the metabolic labelling of viral components could not be performed. IBV isolates are not readily adapted to avian tissue culture systems, particularly those similar to the isolate used in these studies which have been passaged only a limited number of times in embryos (Cunningham, 1975). There has been only one report of the successful use of tissue culture for the study of IBV proteins and the type used in those studies was the highly egg adapted Beaudette strain (Alexander and Collins, 1977).

It has been reported that prior treatment of IBV with phospholipase C' induces haemagglutination activity at low temperatures (Bingham, 1975, Alexander et al., 1976, Alexander and Chettle, 1977). However haemagglutination of purified virus after this treatment was found in the present study to be non-quantitative and irreproducible for the detection of virus. Other laboratories have not used the system as an assay for IBV in analytical studies (Bingham, 1975, MacNaughton and Madge, 1977, Alexander and Collins, 1977). The biochemical basis for haemagglutination induced in IBV particles by phospholipase treatment is not understood.

It is desirable that the techniques and conditions chosen for virus purification should take maximum advantage of the different physicochemical properties which distinguish the virus from any

contaminants of host origin. Centrifugation remains the most powerful tool routinely used for virus purification. All previously published methods for the purification of IBV have involved differential centrifugation at low centrifugal force to remove large particulate cell debris followed by the sedimentation of virus at a high angular velocity. The latter step removes virus from low molecular weight soluble egg proteins which remain in the supernatant. However the extensive use of simple differential centrifugation procedures results in a marked drop in the percentage recovery of infectious virus (Collins et al., 1976).

In the present study precipitation of IBV by the addition of polyethylene glycol (PEG) was investigated as an alternative procedure for the removal and concentration of virus from the bulk of contaminating egg proteins present in the allantoic fluid. The use of PEG prior to further purification has the advantage that high speed differential centrifugation is not required thereby avoiding any mechanical damage to fragile virus particles. There was no significant loss of IBV infectivity after precipitation with 8.7% PEG 6000 in the presence of salt and in addition virus could be concentrated from large volumes of allantoic fluid. Using a similar procedure Heyward et al. (1977) concentrated influenza virus from allantoic fluid with PEG 6000. Approximately 70% of the original infectivity was recovered in the precipitate which was then resuspended in 1/20th of the original volume. The efficiency of the precipitation technique in the present study was much higher than this with 100% infectivity being recovered in a suspension concentrated to 1/200th of the original volume. Wege et al. (1979) also found that the use of PEG 6000 was a suitable method for the

initial concentration of the JHM strain of murine coronavirus from infected tissue culture fluid.

Bingham (1975) purified virus from infected allantoic fluid by pelleting at high centrifugal force (70,000 g for one hour) followed by equilibrium centrifugation in sucrose gradients. Virus was present in at least two bands regardless of the IBV strain examined. Infectivity was associated with both preparations at densities of $1.16 - 1.18 \text{ g cm}^{-3}$ and $1.18 - 1.22 \text{ g cm}^{-3}$ respectively.

Collins et al. (1976) separated egg-grown virus into as many as five distinct populations of infectious virus by a combination of continuous and discontinuous sucrose gradient centrifugation steps. The buoyant densities of these bands varied from 1.14 g cm^{-3} to 1.22 g cm^{-3} . However in a later study employing similar techniques Alexander and Collins (1977) found only one band of infectious virus by sequential use of rate zonal and isopycnic centrifugation. Buoyant densities ranged from $1.16 - 1.20 \text{ g cm}^{-3}$ with a peak at $1.175 - 1.18 \text{ g cm}^{-3}$. In a more recent study, MacNaughton and Madge (1977) also found a single major peak of virus at a density of 1.17 g cm^{-3} in sucrose gradients after isopycnic centrifugation although minor bands of infectivity were occasionally seen at 1.13 g cm^{-3} and 1.22 g cm^{-3} .

After initial concentration the virus was further purified by density gradient centrifugation. In the present study Metrizamide was used routinely as the density gradient material in preference to sucrose. Experiments run in parallel comparing the characteristics of both gradient media demonstrated that the virus reached equilibrium in Metrizamide at low centrifugal force (50,000 g) in a shorter time than in gradients containing sucrose. Infectious

virus had reached equilibrium in Metrizamide gradients after 7 hours centrifugation but required a further 5 hours centrifugation to reach equilibrium in gradients containing sucrose. This is due to the lower viscosity of Metrizamide over the density range required for isopycnic banding (Rickwood, 1978). The conditions of centrifugation were chosen in order to allow a continuous gradient of Metrizamide to form in 6 hours without diffusion of Metrizamide solutions prior to centrifugation.

Only one band of infectivity was found after concentrated virus was further concentrated and purified by isopycnic centrifugation in both Metrizamide and sucrose. The buoyant density of IBV in sucrose was found to be 1.18 g cm^{-3} , a result in agreement with that of Alexander and Collins (1977) and MacNaughton and Madge (1977). Infectious virus possessed a buoyant density of 1.14 g cm^{-3} in Metrizamide gradients, a value similar to that found by Alexander and Collins (1977).

Two consecutive shallow Metrizamide gradients were found necessary in order to separate virus from cellular material. Re-banding in a similar gradient to the first resulted in recovery of virus as a single band at the same buoyant density value (1.14 g cm^{-3}). Electron microscopy of this fraction clearly demonstrated the presence of particles with typical coronavirus morphology. Particles were relatively uniform in appearance with a diameter in the range of 120 to 160 nm. The majority of particles contained evenly spaced projections, many with a complete corona (see Fig. 1a). In addition there was little evidence of penetration by negative stain, in contrast to the toroid appearance of particles prepared by purification in sucrose gradients. The lower osmotic pressure

associated with the use of Metrizamide may therefore preserve the spherical appearance of coronavirus particles. The reduced viscosity of Metrizamide compared with sucrose may also facilitate the preparation of particles with maximum retention of surface projections.

The usefulness of Metrizamide as a gradient material in the purification of other enveloped viruses has been reported. Wunner et al. (1975) showed that Metrizamide permitted the recovery of predominantly rounded pleomorphic particles of respiratory syncytial virus with a clearly defined fringe of projections. In contrast the fringe was absent from a large proportion of particles after centrifugation through sucrose. However not all viruses retain their infectivity in Metrizamide. Measles virus is rapidly inactivated in a 20% w/v Metrizamide solution. In contrast Semliki Forest virus infectivity is not affected by Metrizamide (Vanden Berge et al., 1975). In the present study the infectivity of IBV was shown not to be affected by the presence of Metrizamide; any loss of infectivity during centrifugation may have resulted from frictional forces acting on the virus during sedimentation through the gradient. It was noted in initial experiments that centrifugation procedures employing centrifugal forces in excess of 100,000 g resulted in a marked reduction in the integrity of the outer corona with a corresponding loss of infectivity.

B. Polypeptide Analysis

SDS-PAGE of unlabelled and radiolabelled virus resolved fewer polypeptides than reported previously for IBV. The major components migrated with estimated molecular weights of 90,000, 52,000 and

29,000 respectively (Fig. 8a). SDS-PAGE of iodinated, purified IBV after immunoprecipitation by sera from chickens infected with the IBV isolate used in this study resolved all three major polypeptide peaks correspondingly to molecular weights of 90,000, 52,000 and 29,000. This indicates that these polypeptides are specific components of the virus and unlikely to be contaminants derived from allantoic fluid. The binding of ^{125}I -concanavalin A to the 90,000 and 29,000 molecular weight components indicates the glycoprotein nature of these two polypeptides (Fig. 13). ^{125}I -concanavalin A labelling was used to identify carbohydrate containing polypeptides resolved in polyacrylamide gels rather than periodate-acid Schiff staining. The latter procedure has been shown to be unreliable if SDS is incompletely removed from the gels prior to reduction and staining (Glossman and Neville, 1971). In the absence of 2-mercaptoethanol, an additional glycoprotein was detected in the region of the 50,000 molecular weight polypeptide species together with a smaller 26,000 molecular weight glycoprotein (Fig. 8b). In addition the major polypeptide peak in the region of the 29,000 molecular weight range was absent in those gels run under non-reducing conditions regardless of whether separated components were visualised by Coomassie Blue stain or the sample contained radiolabelled virus. One explanation of these results may be the presence of two rapidly migrating polypeptides in this molecular weight range; one with a molecular weight of 29,000 and a 26,000 molecular weight component which is neither labelled by the Bolton and Hunter reagent nor visualised in gels stained with Coomassie Brilliant Blue. This may possibly arise owing to the high carbohydrate content of this protein. Alternatively there may be only one polypeptide of 29,000 molecular weight which, in the absence of 2-mercaptoethanol migrates mainly as

a dimer with an estimated molecular weight of 50,000 but also exists as a monomer migrating slightly faster at 26,000 molecular weight in comparison with an estimated molecular weight of 29,000 after reduction with 2-mercaptoethanol.

The polypeptide profiles of IBV purified in sucrose gradients were similar to those in Metrizamide (Fig. 5). With both gradient materials two consecutive isopycnic centrifugations were necessary to remove cellular components. This process was monitored throughout by negative staining of fractions followed by electron microscopy. A comparison of SDS-PAGE profiles obtained with virus after one and two consecutive isopycnic bandings demonstrated that high molecular weight components were present after the first centrifugation. However these were removed by the second equilibrium centrifugation and may therefore have been of cellular origin (Fig. 5).

The results reported in the present study for the number and size of polypeptides differ from those reported previously for IBV, which in turn differ from each other (see Table 3).

Despite this extreme variability, there is general agreement as to the presence of a non-glycosylated major polypeptide with an approximate molecular weight of 50,000. This has been shown to be closely associated with the viral nucleocapsid (MacNaughton et al., 1977). A similar polypeptide was found in this study as discussed above. Further experiments showed its close association with a spherical subviral particle released after disruption of IBV with the non-ionic detergent Triton X-100. The high buoyant density of this particle and its high A₂₅₄/A₂₈₀ absorbance ratio suggest that nucleic acid is present and that the 50,000 molecular weight polypeptide represents the ribonucleoprotein as reported by other workers.

Disagreement arise on comparison of both the number and size of the remaining IBV polypeptides. As many as sixteen polypeptides have been reported for IBV (Bingham 1975, Collins et al. 1976, Alexander and Collins, 1977). A majority of these are resolved as minor bands in polyacrylamide gels and their significance is questionable. A comparison of densitometer scans from each publication reveals that the profiles presented by Collins et al. (1976) and Alexander and Collins (1977) agree most closely with those obtained in the present study. Alexander and colleagues found only one major glycopeptide peak in the lower molecular weight range. Their estimated molecular weight of 29,000 for this component is similar to the glycopeptide of 29,000 molecular weight found in the present study. However these authors resolved three glycopentides of higher molecular weights in the region of 53 - 95,000, although the values quoted varied between reports. In the present study only one glycopeptide of high molecular weight was resolved with an estimated molecular weight of 90,000.

MacNaughton et al. (1977) found several major glycopeptides of high molecular weights 130,000, 105,000, 81,000 and 74,000 respectively. However they could resolve only one peptide with a lower molecular weight (33,000) and further analysis showed that this protein was not glycosylated. Recently MacNaughton et al. have revised their estimate of the number of polypeptides in IBV (personal communication) suggesting the presence of only two glycopeptides with molecular weights of 105,000 and 97,000 respectively, the latter appearing as a shoulder on the larger peak.

Suggestions have been put forward that the variation in both the size and the number of polypeptides reported by different workers for coronaviruses may be the result of the different techniques adopted

for virus purification and analysis rather than representing intrinsic differences in their structure. The marked divergence of earlier reports of the structure of IBV from those of other coronaviruses may reflect the difficulty of purifying IBV from allantoic fluid.

Of interests here is that the protein composition of the enteropathogenic bovine coronavirus purified from the intestinal contents of calves differs widely from that of other coronaviruses which are generally purified from tissue culture fluid (Hajer and Storz, 1979) (see Table 2). The method of purification involved differential centrifugation followed by sucrose density centrifugation and was similar to that employed by other laboratories for the purification of IBV. These techniques may be less efficient in removing contaminating egg proteins than those used in the present study, resulting in the large number of minor bands resolved by SDS-PAGE.

The variation among earlier reports (Bingham, 1975, Collins et al., 1976, Alexander and Collins, 1977, MacNaughton and Madge, 1977) in the number and sizes of the polypeptides in IBV is unlikely to arise solely from differences in the techniques used to purify the virus since all laboratories adopted the same separation methods. There were differences however, in the SDS-PAGE systems used by each laboratory in order to analyse the polypeptide composition. The different conditions chosen for sample analysis may therefore, in part, account for the variability in interpreting the protein composition of IBV.

Bingham resuspended pelleted virus in 5% SDS at 37°C for 30 minutes, added 2% 2-mercaptoethanol and then placed the sample in a boiling water bath for 2 minutes. This was dialysed at room

temperature overnight against tris-glycine buffer pH 8.2 containing 1% SDS, 0.1% 2-mercaptoethanol, 3.3 M urea and 5% sucrose. The sample was reheated for 1 minute at 100°C immediately prior to electrophoresis. Gels were prepared in tris-glycine buffer pH 8.9 containing 0.1% SDS and 0.5 M urea and the electrophoresis buffer was tris-glycine pH 7.8 containing 0.1% SDS, 0.5 M urea and 0.1% 2-mercaptoethanol.

The sample preparation techniques used by Collins et al. (1976) and Alexander and Collins (1977) did not involve dialysis. Samples were placed in 0.01 M sodium phosphate buffer pH 7.2 containing 1% SDS and 2% dithiothreitol and placed in a boiling water bath for 2 minutes immediately prior to electrophoresis. The buffer used in the gels and for electrophoresis was 0.05 M sodium phosphate pH 7.2 containing 0.1% SDS and 0.01 M EDTA (Alexander, 1974).

MacNaughton et al. (1977) harvested virus directly from sucrose gradients and added SDS and 2-mercaptoethanol to final concentrations of 5% and 2% respectively. Samples were then heated for 1.5 minutes at 100°C. Gels were prepared in tris-glycine buffer pH 7.8 containing 0.1% SDS and 0.5 M urea, and the electrophoresis buffer consisted of tris-glycine pH 7.8 containing 0.1% SDS, 0.5M urea and 0.1% 2-mercaptoethanol.

It has been suggested that the method of sample preparation prior to SDS-PAGE may markedly influence the number and size of polypeptides resolved. MacNaughton and Madge (1977) showed that up to 12 polypeptides could be resolved if the samples were dialysed overnight against sample preparation buffer containing SDS, 2-mercaptoethanol and urea prior to PAGE. This result compared with

the 7 polypeptides these authors routinely resolved using a procedure which did not involve dialysis. Bingham (1975) routinely dialysed IBV samples overnight against SDS, 2-mercaptoethanol and urea followed by heating at 100°C immediately before electrophoresis. This may therefore account for the large number of polypeptides resolved in that particular study.

Sturman (1977) found an analysis of mouse hepatitis virus that the method of sample preparation influenced the number and size of polypeptides which could be resolved by PAGE. After heating at 100°C in SDS a glycoprotein, previously estimated as possessing a molecular weight of 23,000 aggregated to produce several apparently new polypeptides with a much lower electrophoretic mobility. In addition, Sturman reported that the effects of heating were exaggerated by 2-mercaptoethanol or dithiothreitol.

Pocock (1978) observed no differences in the PAGE profiles of purified HEV regardless of the presence or absence of a reducing agent, suggesting that interpeptide disulphide bonds were not present in the structure. However the migration rate of the 180,000 molecular weight glycopeptide was increased slightly suggesting that an intrapeptide disulphide bond was present.

Wege et al. (1979) also showed that the method of preparation of sample prior to PAGE influenced their results. Boiling of lysed mouse hepatitis virus (JHM strain) with 5% 2-mercaptoethanol for 2 minutes led to strong aggregation of proteins at the top of the gel with a concomitant decrease in the collective amount of peptides with molecular weight: 170,000, 125,000, 24,800 and 22,700. The presence of urea in boiled sample preparations also resulted in aggregation at the top of the gels with a decrease in the smaller

24,800 and 22,700 molecular weight polypeptides. When samples were boiled in the absence of 2-mercaptoethanol and urea there was an additional polypeptide of 40,000 molecular weight present. Similar results were obtained with virus which had been stored for prolonged periods as a dry pellet at -70°C .

The presence of additional bands in gels of samples prepared in buffers containing urea could be due to the reaction of protein with cyanate ions. These are formed by the chemical isomerization of urea at high temperatures and react with amino groups to form very stable carbamylated derivatives. Carbamylated amino groups cannot ionize so the protein charge is altered. A heterogeneous mixture of charged molecules may result which migrate differently to give multiple bands in SDS-PAGE as a result of variable reaction of proteins with cyanate ions (Maizel, 1971).

Particular difficulties are encountered in the analysis of glycosylated proteins owing to their anomalous behaviour in polyacrylamide gel electrophoresis. The presence of carbohydrate affects the formation of SDS-protein complexes (Segrest et al. 1971, Greffrath and Reynolds, 1974) as a result may give variable results. In low concentration polyacrylamide gels, where the rate at which molecules migrate depends almost exclusively on charge, the estimated molecular weight may be significantly lower than the value obtained in gels of higher acrylamide concentration, where separation occurs as a result of molecular sieving (Bretscher, 1971).

The source of SDS may also influence the number of glycopeptides seen in stained gels. SDS from the same source (Sigma Chemical Co.) was used routinely in the present study. Substitution with SDS

obtained from British Drug houses resulted in the failure to resolve the polypeptide band of 29,000 molecular weight in stained gels. In addition ^{125}I -concanavalin A did not bind to the gel in this region indicating the absence of glycopeptide. The failure of PAGE systems containing SDS from British Drug houses to resolve the low molecular weight glycopeptides may reflect the poor binding of SDS from this alternative source to highly glycosylated proteins, thereby altering their charge.

There have been reports of the failure of SDS to dissociate glycoproteins into individual polypeptide chains. This has been shown for two human erythrocyte membrane glycoproteins which appear to migrate in gel electrophoresis in a monomer-dimer relationship (Marton and Garvin, 1973, Tuech and Morrison, 1974). Thus the technique of SDS-PAGE may not be entirely reliable as a method for separating glycopeptides.

Glycoproteins do not stain well with protein stains such as Coomassie Brilliant Blue due to their high carbohydrate content (Carraway et al., 1971) and their presence could remain undetected. Staining of resolved components with periodate-acid Schiff reagent is commonly employed for the detection of glycoproteins. However, this reagent may stain other protein components non-specifically if residual SDS is present in the gels (Glossman and Neville 1971). The staining technique also preferentially detects sialoglycoproteins since sialic acid is the carbohydrate most readily cleaved by periodate treatment. It is possible therefore that glycoproteins with low carbohydrate and no sialic acid may not be detected by this method (Tanner and Boxer, 1972).

Alternative indicator systems for carbohydrate include labelling of gels with ^{125}I -concanavalin A. Concanavalin A binds to α -D-glucopyranosyl, α -D-mannopyranosyl, α -D-fructofuranosyl and

α -D-glucosaminyl. The reaction is inhibited by methyl- α -D-mannopyranoside. Reported methods for binding ^{125}I -concanavalin A to glycoproteins in acrylamide gels required soaking whole washed gels in buffer containing the radiolabelled lectin (DurrIDGE, 1976). However Neurath et al. (1978) found that the sensitivity of the assay could be increased by prior slicing of gels and then immersing each slice in buffer containing ^{125}I -concanavalin A. This increases the surface area of the exposed gel thereby allowing greater quantities of separated glycoproteins to react with the lectin.

In the present study a discontinuous gel/buffer system was chosen. A short stacking gel of large pore and low pH (6.0) was laid over a long (10 cm) resolving gel of high pH (8.7). This system has been used successfully to analyse the polypeptide composition of other enveloped viruses such as Hepatitis B (Skelly et al., 1978) and Pichinde. SDS-PAGE was always performed in the absence of urea. The effect of varying the temperature of sample preparation revealed no difference in the SDS-PAGE profiles of both labelled and unlabelled virus after heating at either 60 $^{\circ}$, 80 $^{\circ}$ or 100 $^{\circ}\text{C}$ (Figs. 10, 12). However when the experiment was repeated in the absence of reducing agent the 29,000 polypeptide peak was absent at all temperatures and the 90,000 molecular weight glycopeptide ran at a slightly higher mobility (Figs. 11, 12). This suggests that the latter component may contain intrapeptide disulphide bonds similar to the 180,000 molecular weight glycopeptide in HEV.

In addition it was noticed that samples reduced with 2-mercaptoethanol gave rise to further minor bands which were barely visible after electrophoresis and staining with Coomassie Brilliant Blue as compared with samples run under non-reducing conditions. The use of dialysis

to remove either sucrose or Metrizamide from samples prior to PAGE analysis was also observed to increase the number of minor bands; these were not seen in gels of samples where procedures such as gel chromatography were used to remove virus from gradient media. Similar observations have recently been reported by MacNaughton et al. (1977).

The significance of minor bands produced in the presence of reducing agent remains in doubt. However the observations concerning the effect of 2-mercaptoethanol on the molecular weight of both the 90,000 and 29,000 glycopeptides help to define the structure of IBV. ¹²⁵I-concanavalin A labelling of acrylamide gels of purified IBV treated with bromelain and run in the presence and absence of reducing agent show that the 90,000 molecular weight glycopeptide is cleaved in at least one position by the enzyme. This glycopeptide, after exposure to bromelain, still migrates with an estimated molecular weight of 90,000 in gels run under non-reducing conditions, probably owing to the presence of intrapeptide disulphide bonds. In the presence of 2-mercaptoethanol, however, the disulphide bonds are reduced and the 90,000 molecular weight component migrates more rapidly as two structures with molecular weights of 60,000 and 40,000 respectively (Fig. 17).

It is interesting to note that the attachment of IBV (Beaudette strain) to chicken kidney cell cultures was found by Lukert (1972) to be inhibited by L-cysteine, reduced glutathione, 1,4-dithiothreitol, 2-mercaptoethanol and p-hydroxymercuribenzoate, the effect of the latter being reversed by L-cystine. This finding further indicates the presence of SH groups exposed on the external surface of IBV virions and the importance of their preservation for full biological activity.

C. Bromelain Treatment

Two approaches were employed in order to determine the location of individual polypeptides with respect to the internal and external components of coronaviruses. The first involved the treatment of intact particles with proteases such as bromelain which may be expected to digest externally situated polypeptides leaving internal components intact. Bromelain is a heterogeneous mixture of enzymes derived from pineapple stem; the major component is a protein with a molecular weight of 28,000.

When bromelain was used to study the structure of IBV all peaks in stained gels were affected (Fig. 15). Treatment with 0.05% bromelain had no effect on the morphology of the virions as visualised by electron microscopy (Fig. 14a) even though SDS-PAGE analysis of treated virus showed that the 90,000 molecular weight polypeptide was missing and the size of the 52,000 and 29,000 molecular weight peaks were much reduced. Several new peaks were resolved (Fig. 15a), which were probably cleavage products of the enzyme-affected native proteins. The retention of viral morphology as revealed by electron microscopy suggests that at this concentration of enzyme viral proteins are cleaved at several positions but not completely removed by protease digestion.

Treatment of intact virus with 0.1% bromelain removed the spikes from virions (Fig. 14b) with concomitant removal of the polypeptides with molecular weights of 90,000, 52,000 and 29,000 respectively (Fig. 15b). On the basis of these results no conclusion could be reached as to the relative positions of polypeptides in the virion structure.

The results reported here of the effect of bromelain on the structure of IBV differ from those reported previously. MacNaughton et al. (1977) found that bromelain removed the surface projections accompanied by the loss of polypeptides with molecular weight 13,000, 105,000 and 74,000. It was concluded therefore that these three polypeptides were components of the surface projections. Bingham (1975), using the same technique suggested that of 16 polypeptides resolved in his analysis of IBV, five of molecular weight 180,000, 130,000, 106,000, 83,000 and 70,000 were accessible to bromelain on the surface of the virion whereas the remaining peptides were internal and therefore not available to the enzyme.

A comparison of these reports obtained with IBV can be made with the effect of bromelain on other coronaviruses. In agreement with studies using IBV, the polypeptide in the 50,000 molecular weight range appears not to be affected by proteases in MHV, TGEV and the human coronaviruses strains 229E and OC43 unless high concentration of enzyme are used. In all examples the removal of projections due to protease digestion could be correlated with the removal of specific polypeptides as detected by SDS-PAGE.

D. Triton X-100 solubilisation of Virus

Several studies have attempted to define the association of viral proteins with morphological components visualised by electron microscopy. The use of non-ionic detergents, such as Triton X-100 and Nonidet P40 (NP40) has been used extensively to solubilise the outer lipid bilayer of enveloped viruses. Components may then be visualised directly on electron microscope grids or separated by techniques such as affinity chromatography or density gradient centrifugation prior to analysis.

Triton X-100 (polyoxyethylene-p-tert-octylphenol) is a mild detergent and most membrane enzymes retain activity in its presence (Helenius and Soderlund, 1973). The kinetics of its reaction with the membrane of Semliki Forest virus were studied by Helenius and Soderlund (1973) using labelled detergent. They found that the solubilisation of the envelope occurred by a stepwise mechanism progressing 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. Final and complete solubilisation of lipid was associated with the production of protein-detergent complexes. The disruption products at each stage were separated on sucrose density gradients and analysed by electron microscopy and SDS-PAGE. The nucleocapsid was released at low Triton X-100 concentrations which disorganised the structure of the membrane. Complete solubilisation of the lipid was achieved at concentrations of Triton X-100 of less than 1% w/v.

Similar studies with vesicular stomatitis virus (Wunner et al. 1975) showed that the salt concentration affected the nature of the subviral particles dissociated after solubilisation of the lipid envelope in Triton -N101. In low ionic strength buffers a 'skeleton' of the virus was produced which lacked the lipid envelope and the surface membrane glycoproteins. However, in the presence of high salt concentrations, the matrix protein was also lost, leaving only virion RNA, the nucleocapsid protein (N) and the L and NS proteins which are involved in replication.

MacNaughton et al. (1977) disrupted purified IBV with 1.0% Nonidet P40 in low salt buffer and separated virion components by sucrose density centrifugation. The resulting fractions were

characterized by PAGE. They were unable to isolate the 50,000 molecular weight component but did recover disrupted virions which were penetrated by stain and apparently lacked the internal component. No information about the sedimentation characteristics of these particles was noted. SDS-PAGE profiles of NP40 treated particles were similar to those of untreated virus except that the amount of the 50,000 molecular weight component resolved by PAGE was considerably reduced. MacNaughton et al. therefore suggested that the 50,000 molecular weight component is the ribonucleoprotein which is removed together with the core component by solubilisation of the outer envelope with Nonidet P40.

A separate attempt to characterize the internal component of IBV was made by Bingham and Almeida (1977). Nonidet P40 in low salt buffer was similarly used to disrupt virions and the solubilised components separated by sucrose gradient centrifugation. Only one band was detected in sucrose gradients at a density of $1.27 - 1.29 \text{ g cm}^{-3}$. Unfortunately, insufficient quantities of protein prevented SDS-PAGE analysis of this component. Electron microscopy of this band did reveal, however, the presence of aggregated membrane-like structures. The continuing presence of membrane in NP40 treated virus in both this study and that of MacNaughton et al. (1977) suggests that the particles had not been completely solubilised with the detergent at the concentrations selected.

Attempts were made in the present study to recover virion components after solubilisation by several different procedures. The first technique investigated of separating viral components after solubilisation in Triton X-100 was Con - A chromatography. The method used was essentially as detailed by Hayman et al.

(1973); influenza virus was solubilised in sodium deoxycholate and the reaction mixture chromatographed on a column of Lens culinaris phytohaemagglutinin conjugated to Sepharose 4B previously equilibrated in buffer containing the detergent. In the present study Triton X-100 was used to solubilise virus and the affinity chromatography was carried out using Con A-Sepharose instead of Lens culinaris lectin. Only labelled virus was used in these experiments. Figure 18 shows that most of the radioactivity (67%) was eluted with the void volume and very little (2%) was eluted by methyl mannoside although previous experiments had shown that most of the major proteins of IBV are glycosylated. A large proportion (31%) was not recovered from the column. The difficulty of performing reliable SDS-PAGE analyses and the confusing results indicated that techniques such as isopycnic centrifugation might prove more suitable for the separation of virion components.

The purification procedure performed as previously described allowed the production of unlabelled virus at high concentrations suitable for disruption with non-ionic detergents. The components were then separated by equilibrium centrifugation in either sucrose or Metrizamide gradients and subsequently analysed by both SDS-PAGE and electron microscopy. Two bands of protein were obtained in sucrose gradients at buoyant densities of 1.23 g cm^{-3} and greater than 1.32 g cm^{-3} . SDS-PAGE analyses showed that the denser band contained a non-glycosylated polypeptide of 52,000 molecular weight together with a glycopeptide of 26,000 molecular weight. Electron microscopy of this material revealed the presence of amorphous aggregates.

Two bands of protein were similarly obtained in Metrizamide gradients at buoyant densities of 1.22 g cm^{-3} and greater than 1.32 g cm^{-3} . SDS-PAGE analysis of the dense material gave results identical to those obtained for the corresponding band in sucrose. However, when visualised by electron microscopy this dense band was found to contain aggregates of spheres 25-45 nm in diameter (Fig. 29). The A254/A280 absorbance ratio of this dense band was approximately 2 as compared to 1 for the lighter material, indirectly indicating the presence of nucleic acid, a finding consistent with its high buoyant density. These spherical structures may therefore represent the isolated cores of IBV virions.

The observation that the dense subviral particle retained its spherical shape in Metrizamide but not sucrose probably reflects the difference in osmolarity between the two gradient materials and may explain why earlier attempts to define the internal structure of IBV were not successful. (MacNaughton et al., 1977, Bingham and Almeida, 1977).

Similar results have been reported for HEV (Pocock and Garwes, 1977) TGEV (Garwes et al. 1976) and MHV (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 sucrose density gradient centrifugation. Spherical structures were consistently isolated which contained RNA associated with a non-glycosylated polypeptide of approximately 50,000 molecular weight. In addition at least one glycopeptide of molecular weight 26,500 in HEV and 24,000 in MHV was present in these examples. Two glycopeptides have been found to be associated with the core of TGEV with

molecular weights of 30,000 and 28,500 respectively. The size of the spherical particles obtained was found to vary among the different coronaviruses.

The existence of subviral particles containing a glycopeptide associated with a non-glycosylated polypeptide and nucleic acid has been reported for viruses other than coronaviruses. Ramos et al. (1972) showed that the arenavirus, Pichinde could be solubilised in NP40 with the release of a fast sedimenting particle which contained viral RNA, a polypeptide of 72,000 molecular weight and a glycopeptide of similar size. This particle banded at a density of 1.32 - 1.34 g cm^{-3} in CsCl.

The subviral particles which banded at a buoyant density of 1.23 g cm^{-3} in sucrose and 1.22 g cm^{-3} in Metrizamide were visualised in electron micrographs as spike-like structures 16 nm in length and probably represent the spikes of the outer corona, Figs. 24, 28. Resolution was not sufficient to distinguish in detail either the structure of the spikes or the presence of multiple morphologies. SDS-PAGE of this material resolved polypeptides 90,000, 52,000 and 29,000, the latter being absent in gels run under non-reducing conditions.

These results suggest the existence in IBV of two glycoproteins in the lower molecular weight region. One of 29,000 molecular weight is linked to itself or another polypeptide by disulphide bonds to form a larger peptide and exists on the surface of the virion where it can be radiolabelled by the Bolton and Hunter reagent. The other has a molecular weight of 26,000 and is not labelled by the Bolton and Hunter reagent possibly because it has not free amino groups external to the lipid bilayer.

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The presence in the virion of two non-glycosylated polypeptides migrating in the 52,000 molecular weight range may help to explain why bromelain reduces the amount of this polypeptide, the component external to the 'core' being susceptible to the enzyme. The observation that no radiolabel sediments with the 'core' structures when iodinated IBV is solubilised with unlabelled virus in Triton X-100 and then centrifuged to equilibrium in Metrizamide gradients (Fig. 27) indicates that the Bolton and Hunter reagent does not penetrate to these structures. The SDS-PAGE profiles of radiolabelled proteins comprising the lighter band (Fig. 30) show that the proportions of label in each peak are similar to those of label in untreated labelled virus (Fig. 10). This is further evidence that there are two polypeptides with molecular weights of 52,000, only the component external to the core being accessible to Bolton and Hunter reagent.

Alternatively there may be only one 52,000 molecular weight polypeptide, a proportion of the total number of copies migrating with the projections whilst the residue remains bound to the 'core' during exposure to Triton X-100. This implies that bromelain and Bolton and Hunter reagent can penetrate the envelope. Further experiments such as tryptic peptide mapping of these two polypeptides would be necessary to determine if they are indeed discrete viral proteins.

The results obtained in this study suggest that the protein structure of IBV may resemble that which has been described for coronaviruses pathogenic for mammals. The large number of bands previously seen in stained gels of purified IBV may in part result

from inadequate purification techniques. Alternatively they may not represent distinct polypeptides but may merely result from either cleavage or aggregation of original polypeptides during sample preparation prior to SDS-PAGE. The presence of reducing agents have been shown to alter the polypeptide profiles and the presence of urea may result in a heterogeneous mixture of electrophoretic components due to the accumulation of cyanate ions derived from the isomerization of urea at high temperatures thus giving rise to additional bands (Maizel, 1971).

Perhaps the greatest difference between polypeptide profiles of IBV obtained in this study and other coronaviruses is the absence of the large molecular weight species in the 180,000 region. However Sturman (1977) has suggested that the 180,000 molecular weight component of mouse hepatitis virus (MHV) contains amino-acid sequences common to a smaller 90,000 molecular weight component. This latter polypeptide may therefore be a cleavage product of a higher molecular weight component.

Conclusion

From the information presented in the discussion above an attempt may be made to describe the structure of infectious bronchitis virus (see Fig. 31). The nucleic acid is associated with a 'core' structure 25-45 nm in diameter. The major polypeptide associated with the 'core' is a non-glycosylated peptide with a molecular weight of 52,000.

The 'core' may be released by solubilising virus in non-ionic detergents. When density gradient centrifugation is used to separate subviral particles a glycoprotein with a molecular weight of 26,000 migrates with the 'core' and the 52,000 molecular weight polypeptide. The association of this glycopeptide with the 'core' protein may occur in whole virus. Alternatively the association may be an artifact arising from the experimental conditions. Sturman (1978) in a similar study with MHV strain A59 found that a glycoprotein with a molecular weight of 23,000 sedimented with the nucleocapsid only when whole virus was solubilised in non-ionic detergent at 37°C. If the experiment was performed at 4°C the glycopeptide sedimented with the other membrane proteins. No convincing explanation has been advanced to explain this phenomenon. In studies with HEV (Pocock and Garwes, 1977), TGEV (Garwes et al. 1976) and MHV strain JHM (Wege et al. 1979) the temperature of incubation did not influence the results. In the present study all incubations were carried out at 37°C. The glycopeptide with a molecular weight of 26,000 was not detected in any other fraction of the density gradient.

A non-glycosylated polypeptide with a molecular weight ^{of 52,000} ~~was~~ also observed to sediment with the 90,000 and 29,000 glycopeptides in a band of protein which was less dense than the 'cores'. This may have been 'core' protein removed by Triton treatment. Alternatively it may occur in the virion as a separate polypeptide external to the 'core', possibly as a matrix protein. A proportion, at least, is accessible to bromelain and Bolton and Hunter reagent. Experiments to determine whether or not there are two polypeptides with a molecular weight of 52,000 were not undertaken in the present

study and would require techniques such as tryptic peptide mapping.

The 90,000 and 29,000 molecular weight glycopeptides 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 molecular weight polypeptide.

Figures 15 and 16 suggest that the 90,000 molecular weight component is almost completely digested by bromelain treatment while the 29,000 molecular peptide is only mildly digested. This may reflect a difference in susceptibility to the enzyme: the former comprising the projections and being more accessible than the latter which is protected by the envelope.

Further studies are required in order to define the relationship of these components and the intracellular events accompanying coronavirus maturation. Relevant investigations, however, may not be possible until the development of more suitable tissue culture systems for infectious bronchitis virus.

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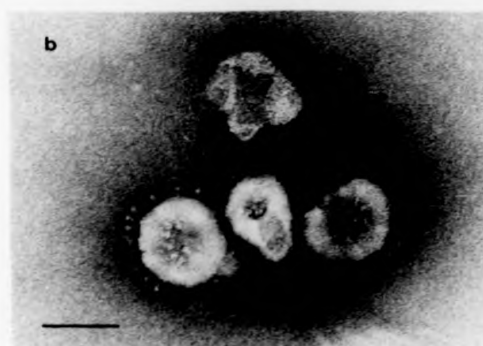
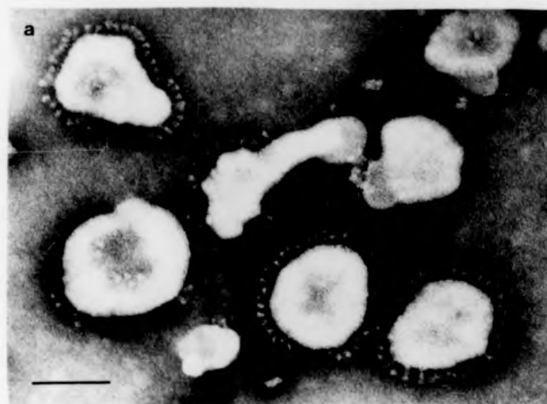


Figure 1

Electron micrograph of IBV stained with potass.phosphotungstate pH 6.0. Virus was purified by PEG precipitation followed by two consecutive isopycnic centrifugations in (a) Metrizamide; (b) Sucrose. Bar represents 100 nm.

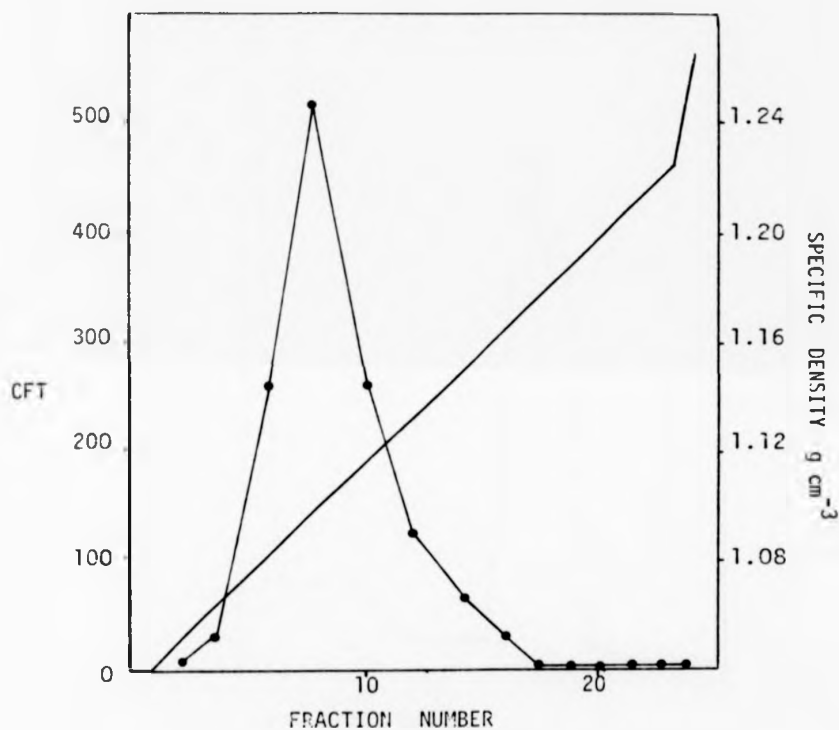


Figure 2

Distribution of IBV complement-fixing antigen in a 5-45% Metrizamide gradient. Centrifugation was for 7 hours at 60,000 \times . The sample consisted of 4 ml of virus precipitated from infected allantoic fluid by the addition of PEG 6000 and resuspended in GNT buffer. IBV infectivity was present as a single band with a buoyant density of 1.14 g cm^{-3} .

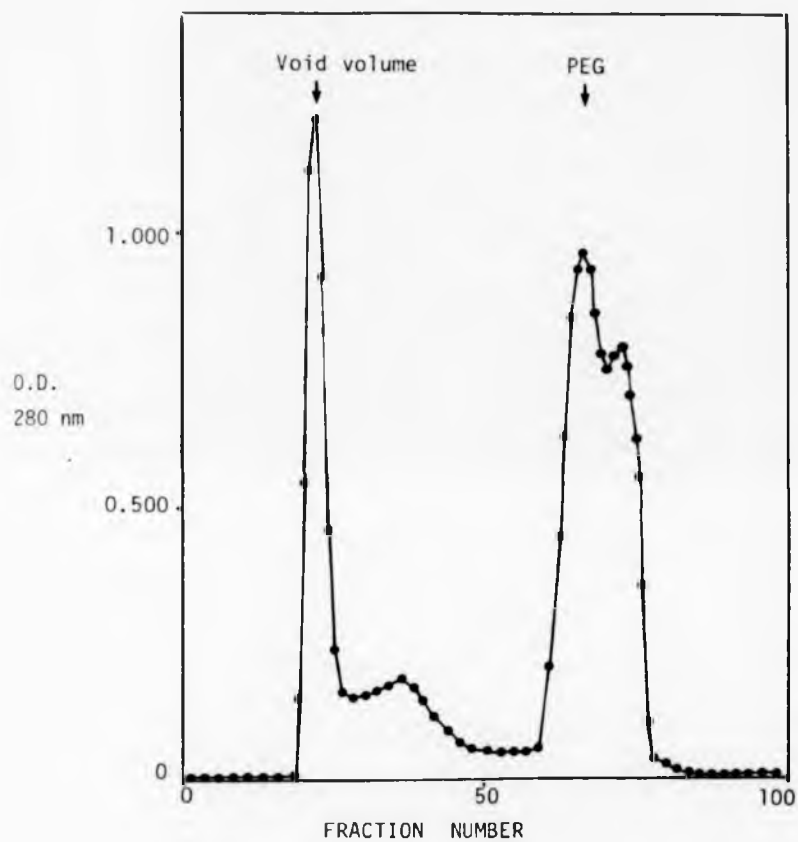


Figure 3

Gel chromatography of PEG precipitate from infected allantoic fluid through a 2.6 cm x 30 cm column of Biogel A-5m equilibrated in GNT buffer. IBV infectivity was present in the void volume fractions and PEG 6000 was eluted in the second peak.

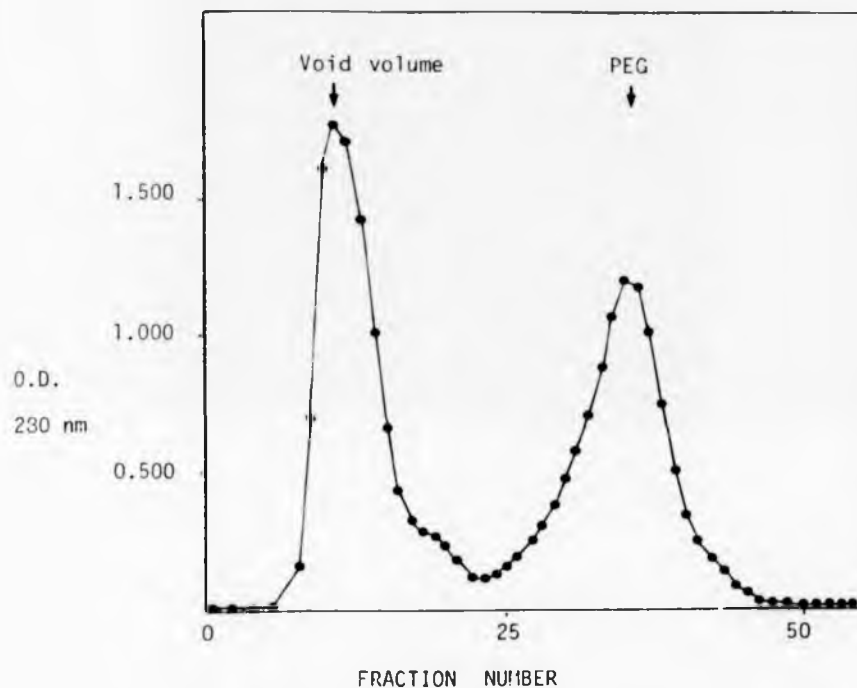


Figure 4

Gel chromatography of PEG precipitate from infected allantoic fluid through a 1.5 cm x 20 cm column of Sephadex G-75 equilibrated in GITE buffer. IDV infectivity was present in the void volume fractions and PEG 6000 was eluted in the second peak.

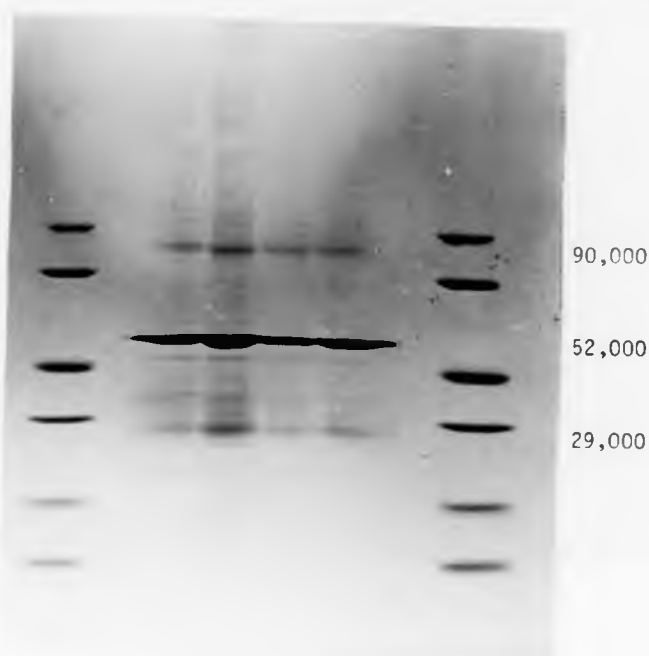


Figure 5

SDS-polyacrylamide slab gel electrophoresis of purified IBV in a 5-15% acrylamide gradient slab gel. Slots 1 to 6 contained standard proteins of known molecular weight (from top) 93,000, 69,000, 43,300, 30,000, 20,000 and 14,300. Slot 2 contained IBV after one isopycnic centrifugation in Metrizamide; slot 3 contained IBV after one isopycnic centrifugation in sucrose; slots 4 and 5 contained IBV after two consecutive isopycnic centrifugations in Metrizamide and sucrose respectively. Note the presence of high molecular weight bands in virus which had been centrifuged to equilibrium once only. Samples were prepared prior to SDS-PAGE in disruption buffer containing 2% SDS and 5% 2-mercaptoethanol and heated at 100°C for 2 minutes. Protein bands were stained with Coomassie Brilliant Blue.

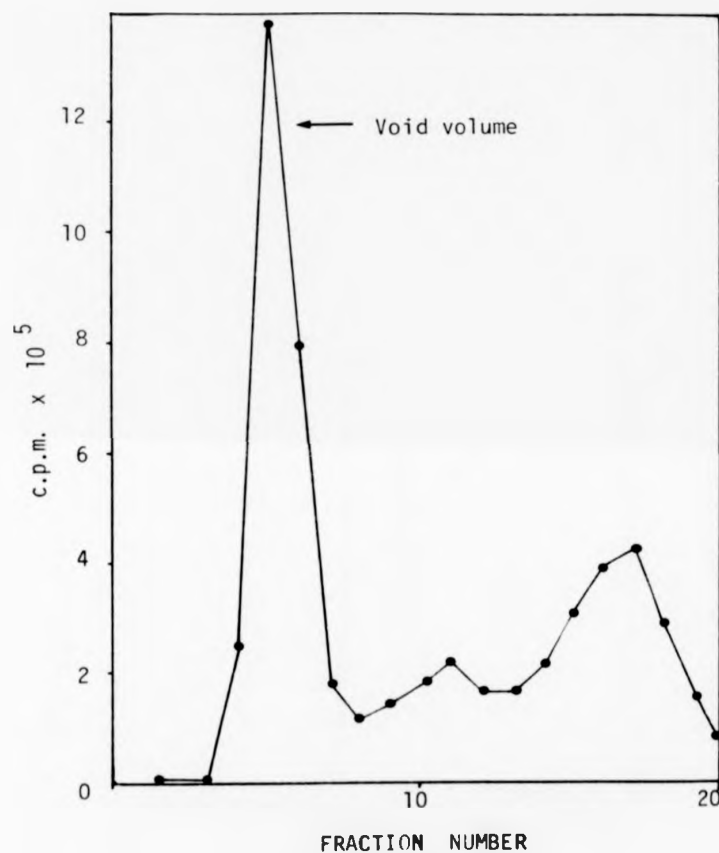


Figure 6

Gel chromatography of purified IBV radiolabelled by the Chloramine-T technique. After radio-iodination the reaction mixture was chromatographed on a pre-packed Sephadex G-25 column PD-10, Pharmacia Fine Chemicals equilibrated in PBS containing 0.25% gelatin. Radio-labelled IBV was present in the void volume fractions.

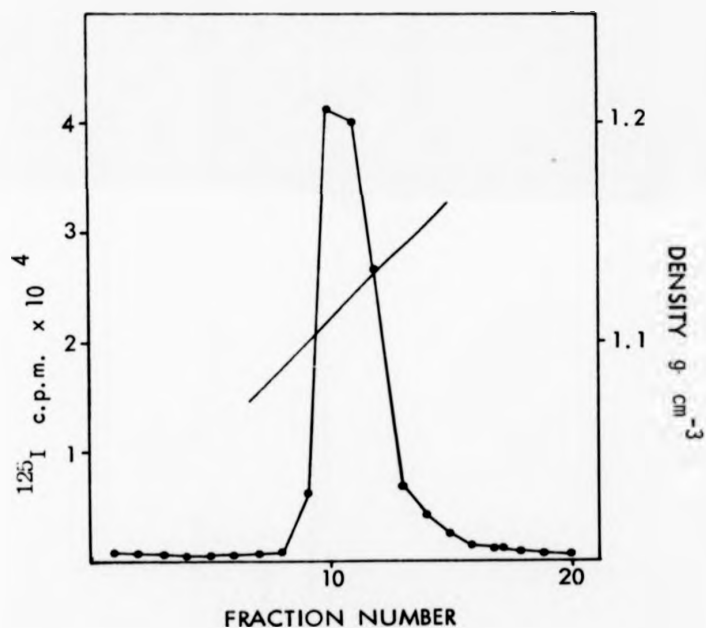


Figure 7

Sedimentation profile of ^{125}I odinated IBV in Metrizamide gradients after 3 hours centrifugation at 50,000 g in a Beckman SW40 rotor. Sedimentation was from left to right. IBV had been radiolabelled by the method of Bolton and Hunter.

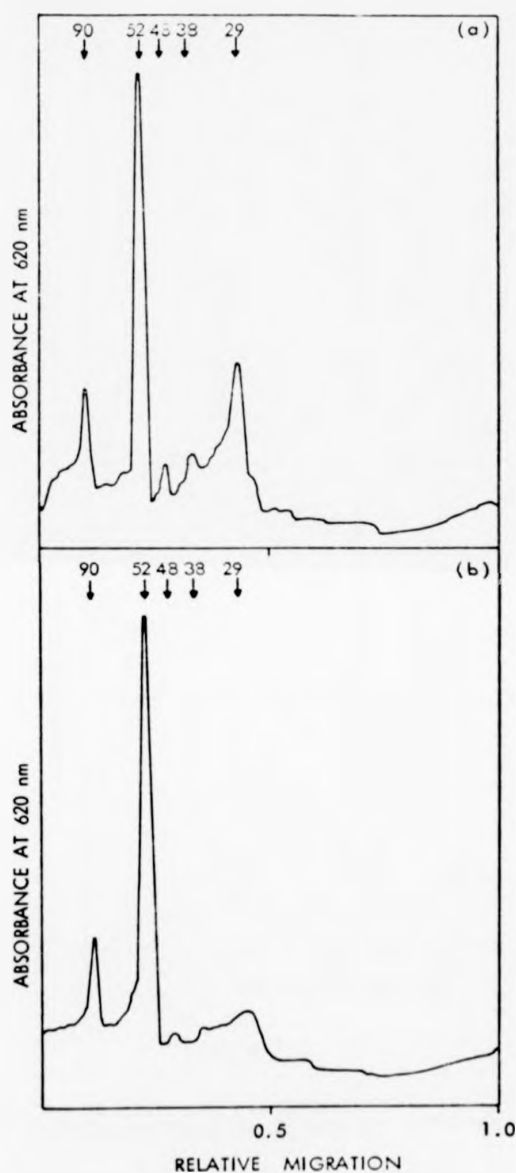


Figure 8

SDS-polyacrylamide disc gel electrophoresis in 10% gels of purified IBV solubilised in 2% SDS at 100°C for 2 minutes: (a) in the presence of 5% 2-mercaptoethanol; (b) in the absence of 2-mercaptoethanol. Protein bands were stained with Coomassie Brilliant Blue. Note the reduction in the size of the peak corresponding to a molecular weight of 29,000 when SDS-PAGE was performed in the absence of 2-mercaptoethanol.

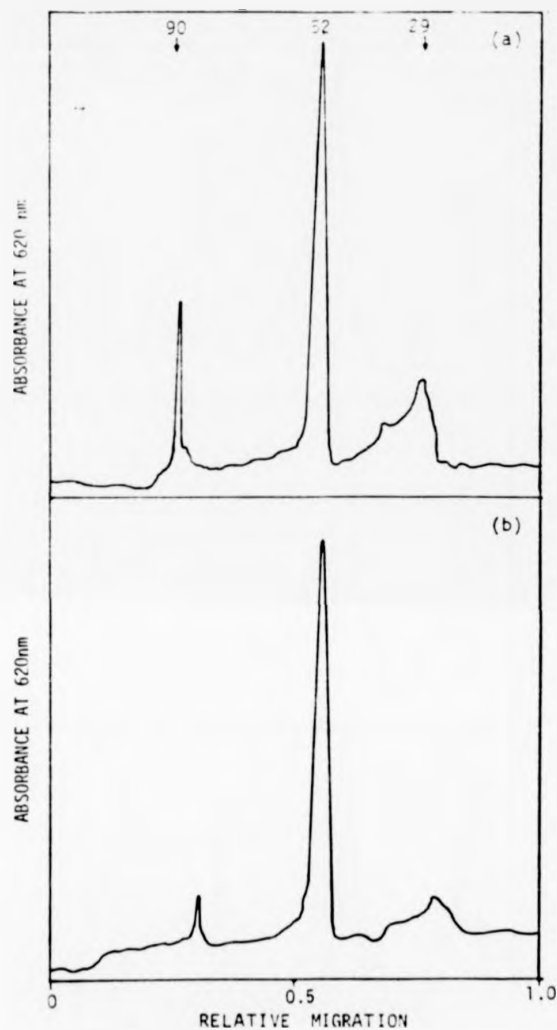


Figure 9

SDS-polyacrylamide disc gel electrophoresis in 7.5% gels of purified IBV solubilised in 2% SDS at 100°C for 2 minutes:

- (a) in the presence of 5% 2-mercaptoethanol,
- (b) in the absence of 2-mercaptoethanol.

Protein bands were stained with Coomassie Brilliant Blue.

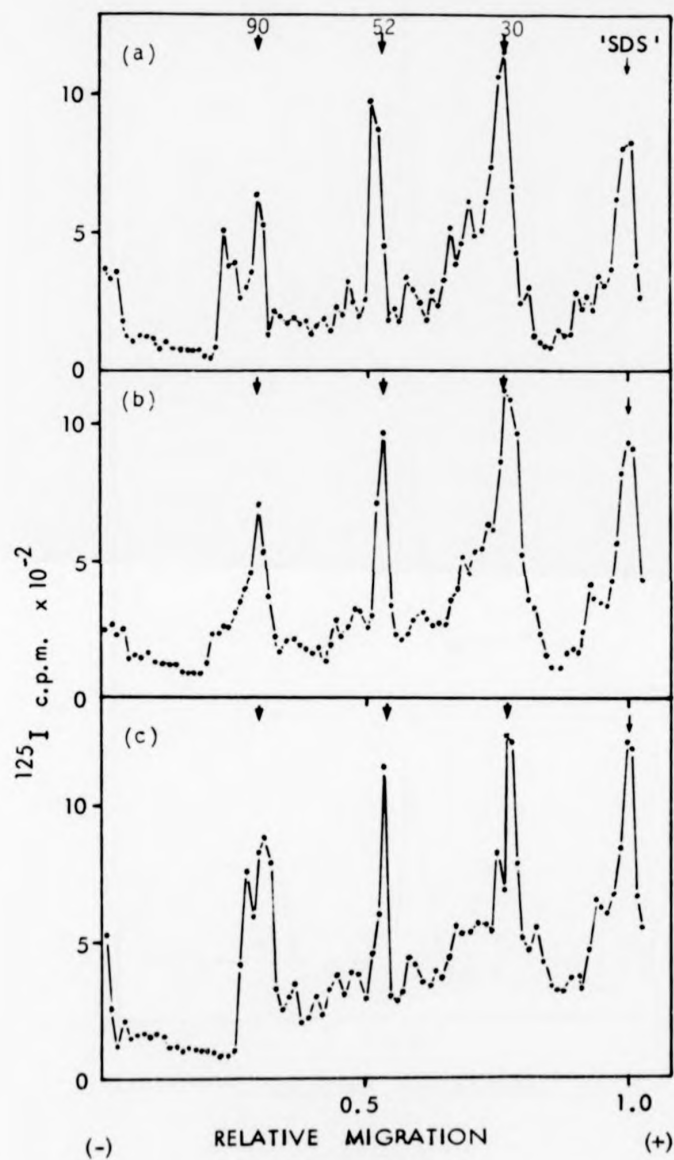


Figure 10

SDS polyacrylamide disc gel electrophoresis in 7.5% gels of ¹²⁵Iodinated IBV particles solubilised in 2% SDS and 5% 2-mercaptoethanol at three temperatures:

- (a) 60°C for 1 h.,
- (b) 30°C for 10 min. and
- (c) 100°C for 2 min.

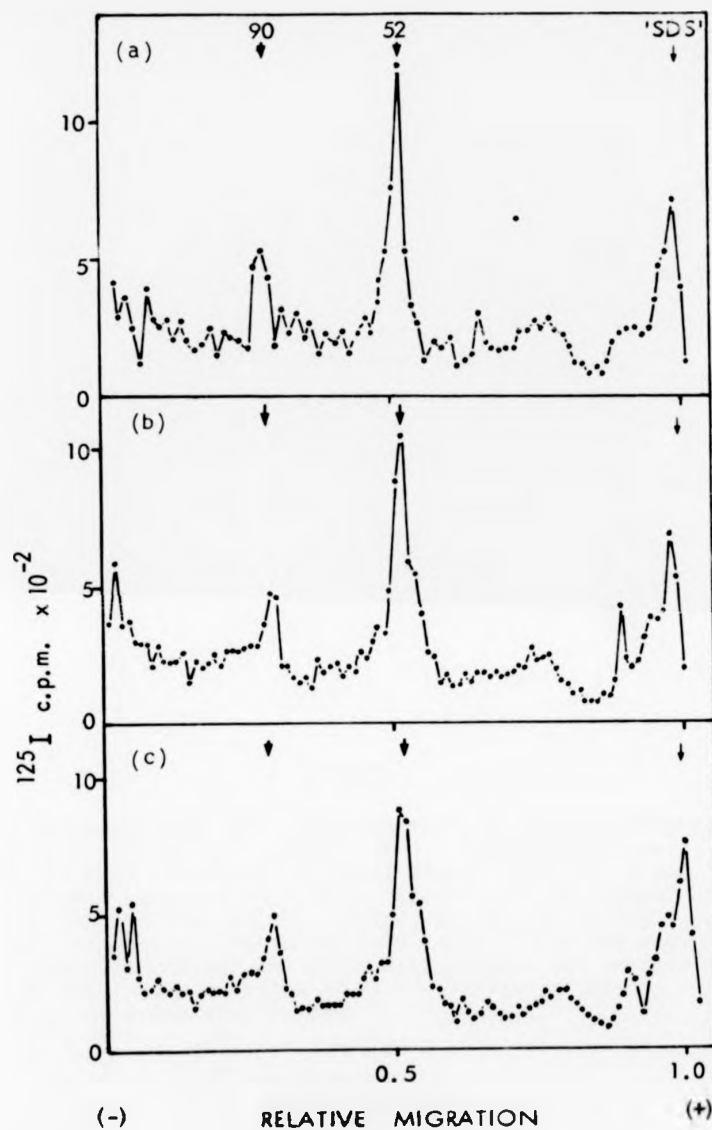


Figure 11

SDS-polyacrylamide gel electrophoresis in 7.5% gels of ^{125}I iodinated IBV solubilised in 2% SDS in the absence of 2-mercaptoethanol at:

- (a) 60°C for 1 h.,
- (b) 80°C for 10 min. and
- (c) 100°C for 2 min.



Figure 12

SDS polyacrylamide gel electrophoresis in 5-15% acrylamide gradient slab gel of purified IBV. Protein bands were stained with Coomassie Brilliant Blue. Slots 1, 2 and 13 contain standard proteins of known molecular weight (from top) 93,000, 69,000, 43,000, 25,500 and 14,300. Slots 3-7 contain IBV solubilised in 2% SDS in the absence of 2-mercaptoethanol and slots 8-12 contain IBV solubilised in 2% SDS in the presence of 5% 2-mercaptoethanol. Disruption temperatures were:

slots 3, 8 : 25°C for 3 hours;
 4, 9 : 37°C for 2 hours;
 5, 10 : 60°C for 1 hour;
 6, 11 : 80°C for 10 minutes;
 7, 12 : 100°C for 2 minutes.

Note the slightly faster mobility of the band with a molecular weight of 90,000 and the reduction in the staining intensity of the band with a corresponding molecular weight of 29,000 in the absence of 2-mercaptoethanol. Note also the effect on the profiles of 2-mercaptoethanol diffusing from slots 2 and 8 to adjacent slots 3, 4, 6 and 7 which did not contain reducing agent.

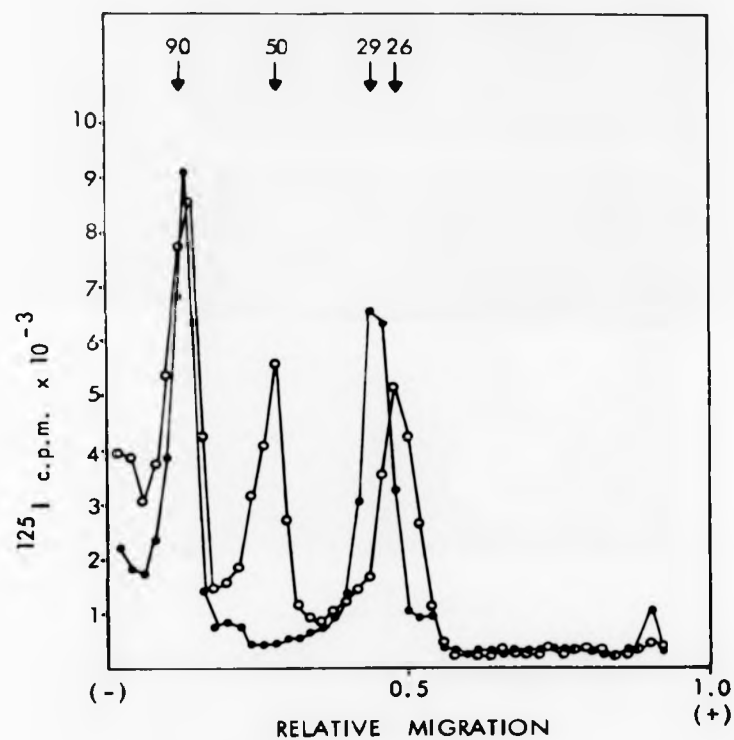


Figure 13

Identification of IBV glycoproteins. Profile of radioactivity obtained after incubating ^{125}I -concanavalin A with sliced 10% polyacrylamide gels of purified IBV, which had been solubilised in 2% SDS:

- in presence of 5% 2-mercaptoethanol
- in absence of 2-mercaptoethanol.

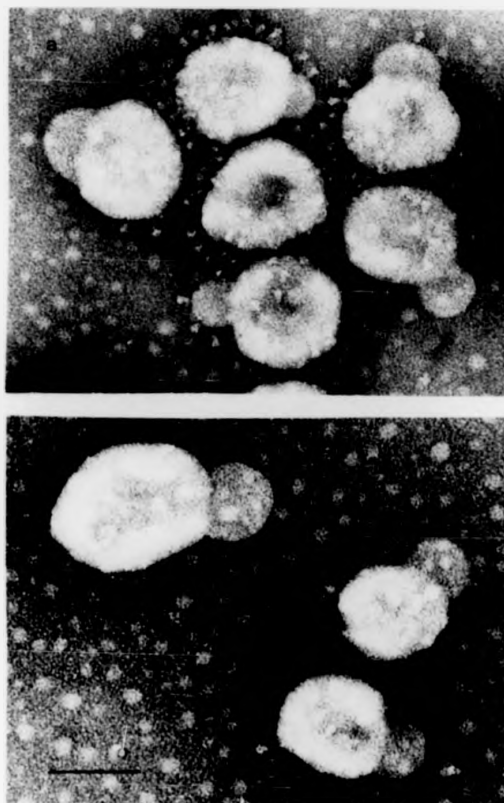


Figure 14

Electron micrograph of purified IBV banded in a Metrizamide gradient after being treated for 1½ hours at 37°C with

(a) 0.05 % bromelain

(b) 0.1% bromelain.

Bar represents 100 nm.

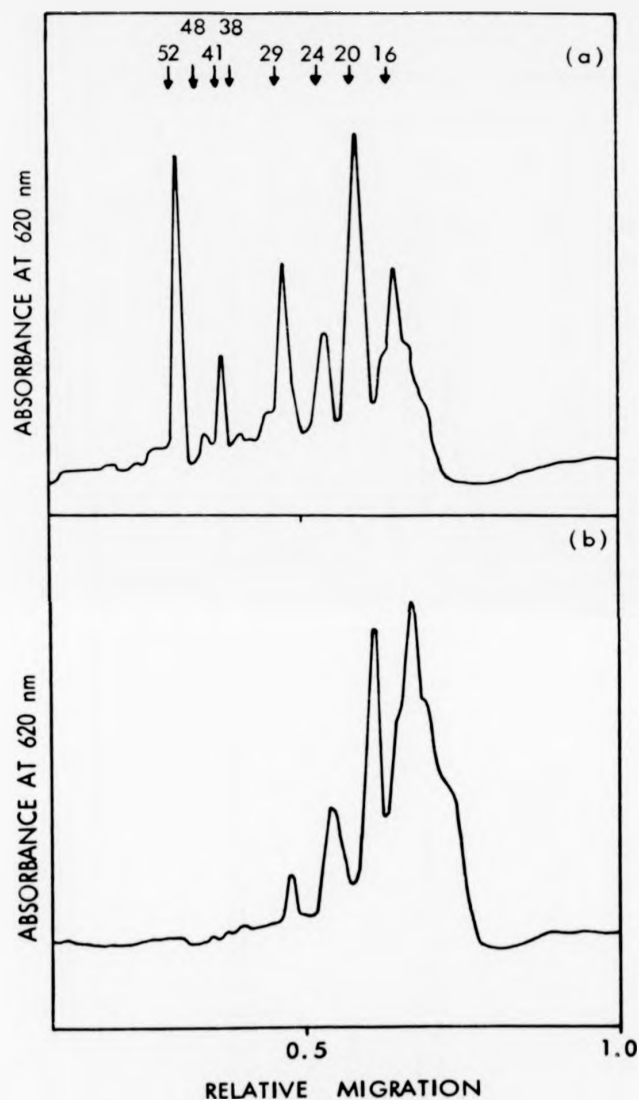


Figure 15

SDS polyacrylamide gel electrophoresis in 10% gels of virus particles which had been subjected to equilibrium centrifugation in Metrizamide gradients after being heated for 1½ h. at 37°C in:

- (a) 0.05% bromelain and
- (b) 0.1% bromelain.

Samples for electrophoresis were solubilised in 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 minutes. Protein bands were stained with Coomassie Blue.

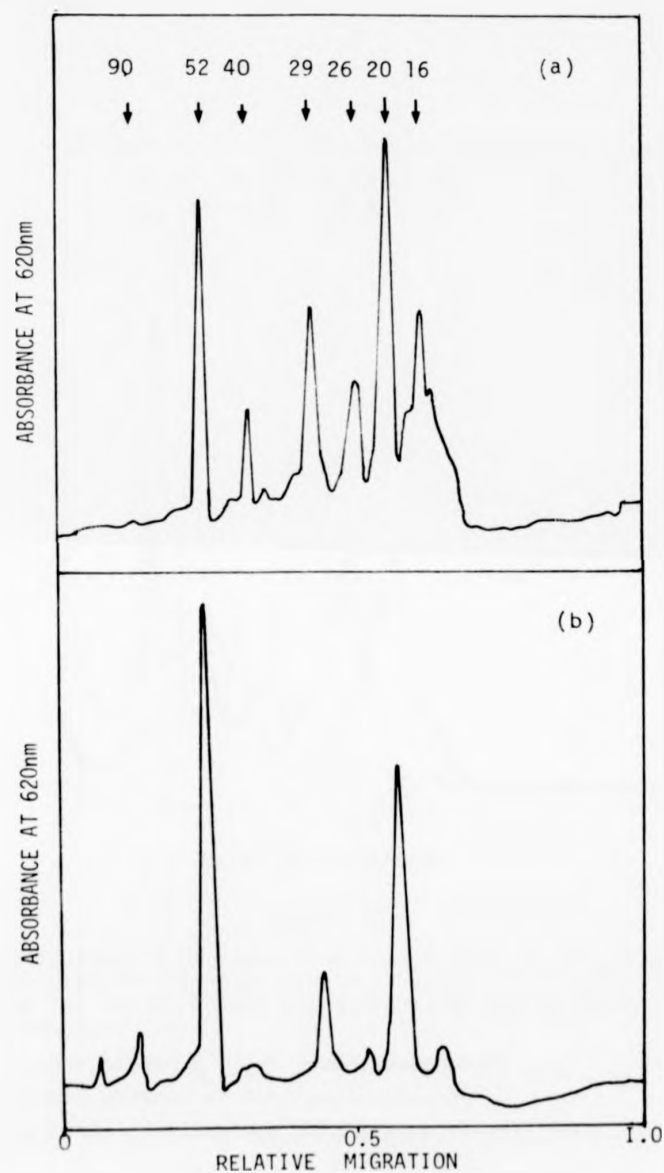


Figure 16

SDS-polyacrylamide gel electrophoresis in 10% gels of virus particles which had been subjected to equilibrium centrifugation in Metrizamide gradients after being heated for 11 hours at 37°C in 0.5% bromelain. Samples for SDS-PAGE were disrupted in 2% SDS at 100°C for 2 minutes in: (a) the presence of 2-mercaptoethanol. (b) the absence of 2-mercaptoethanol. Their positions were indicated with Cassie Brilliant Blue.

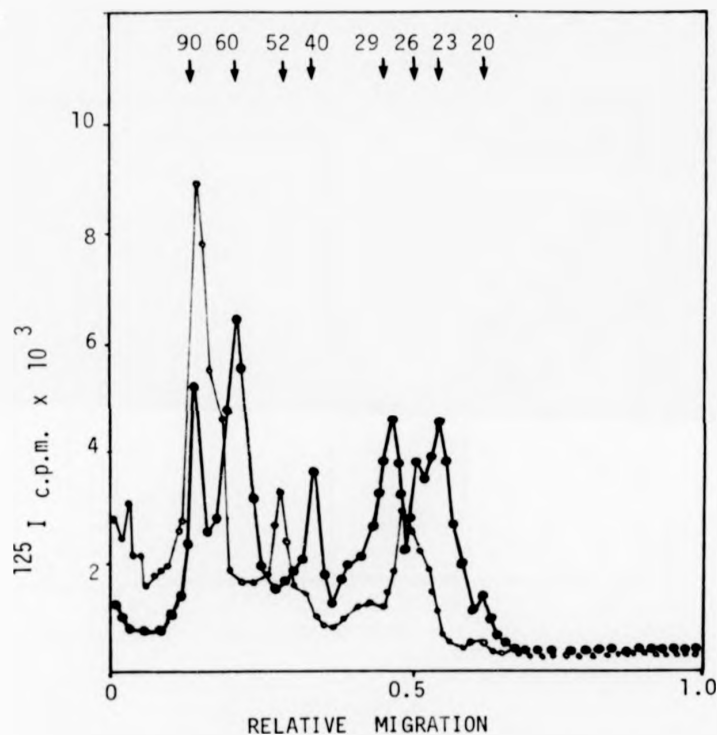


Figure 17

Identification of glycoproteins in purified IBV which had been treated for 1½ hours with 0.5% bromelain at 37°C. Samples for SDS-PAGE were disrupted in 2% SDS at 100°C for 2 minutes:

● in the presence of 5% 2-mercaptoethanol

○ in the absence of 2-mercaptoethanol.

Protein bands were stained with Coomassie Brilliant Blue.

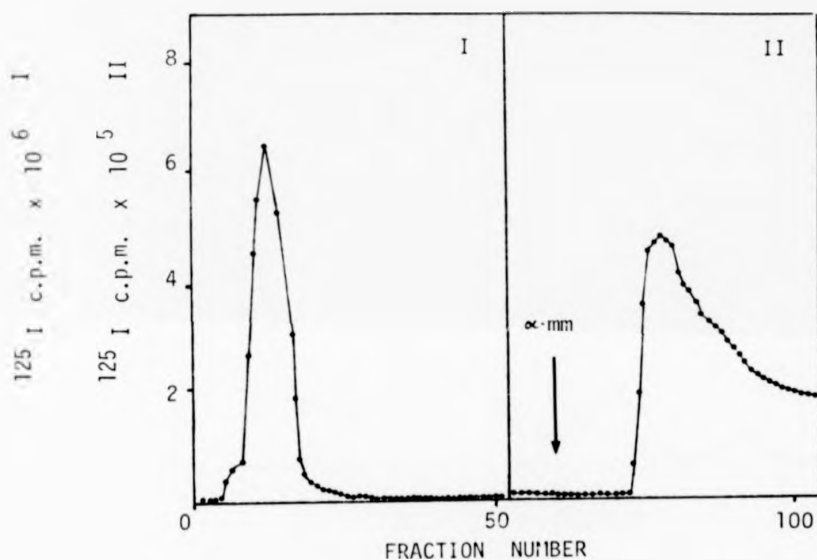


Figure 13

Affinity chromatography of Triton X-100 disrupted ^{125}I -IBV. ^{125}I -IBV was solubilised in 2% Triton X-100 in GTE buffer containing 0.5M NaCl for 4 hours at 37°C . Treated virus was then applied to a 0.9×10 cm column of Con canavalin A-Sepharose and washed with 0.1M tris-HCl buffer containing 0.5M NaCl, 2% Triton X-100, 1mM CaCl_2 and 1mM MnCl_2 (Peak I). Adsorbed radioactivity was eluted by the addition of tris buffer containing NaCl and α -methylmannoside (Peak II).

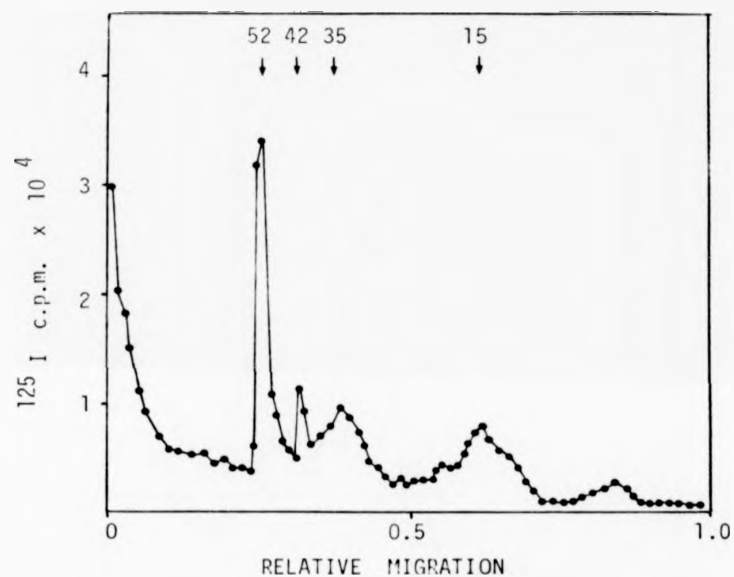


Figure 1

SDS-polyacrylamide disc gel electrophoresis in 10% gels of unbound fraction (peak 1, figure 1b) obtained by affinity chromatography of 125I-labeled protein solubilised in 2% Triton X-100 on Concanavalin A-Sepharose. The sample for SDS-PAGE was disrupted in 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 minutes.

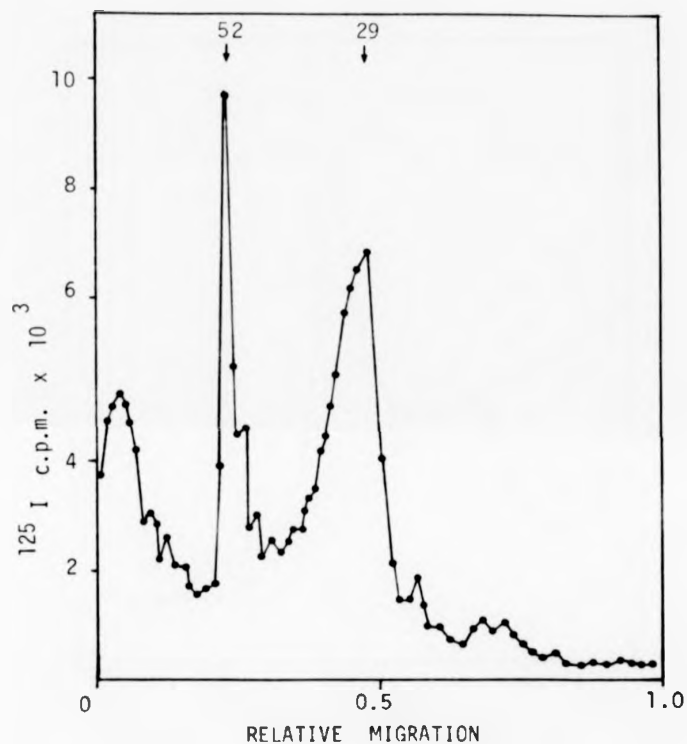


Figure 20

SDS-polyacrylamide disc gel electrophoresis in 10% gels of bound fraction (peak II, figure 18) obtained by affinity chromatography of ^{125}I -IBV solubilised in 2% Triton X-100 (on Concanavalin A-Sepharose). The sample for SDS-PAGE was disrupted in 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 minutes.

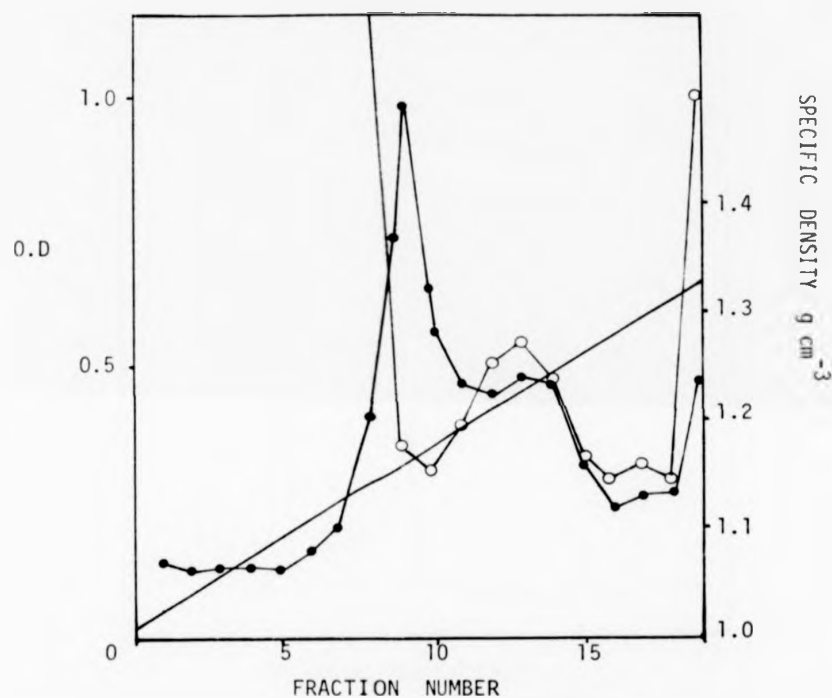


Figure 21

Sedimentation in a linear sucrose gradient of purified IBV solubilised in 2% Triton X-100. Solubilised virus was placed on 20-65% w/w sucrose gradient and centrifuged for 24 hours at 150,000 g in a Sorvall AH-650 rotor. The gradient was then fractionated into 0.25 ml aliquots and the absorbance of each fraction was determined at 230 nm (●) and 254 nm (○).

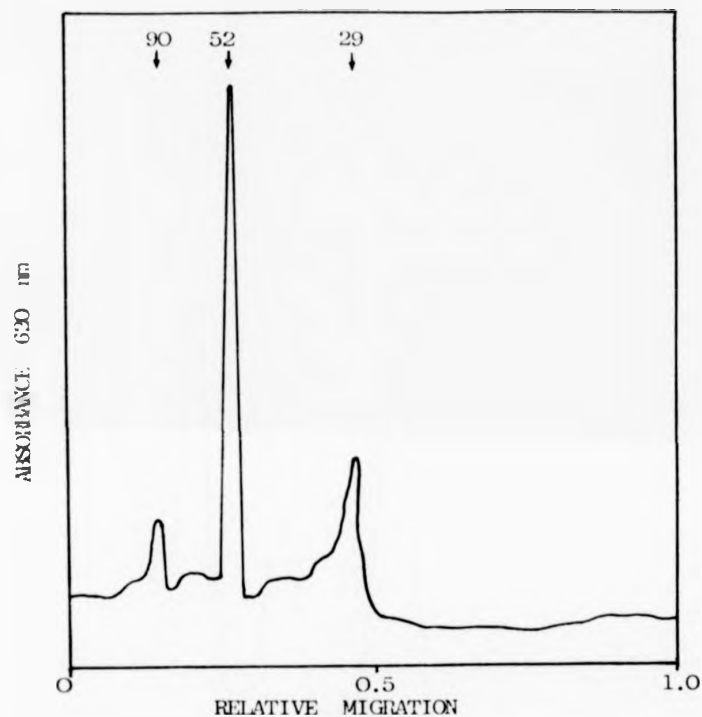


Figure 22

SDS-polyacrylamide disc gel electrophoresis in 10% gels of the low density band of protein (1.23 g cm^{-3}) in sucrose gradient containing IBV solubilised in 2% Triton X-100. Protein bands were dialysed against GNTG buffer for 6 hours and disrupted in 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 minutes prior to electrophoresis. Protein bands were stained with Coomassie Brilliant Blue.

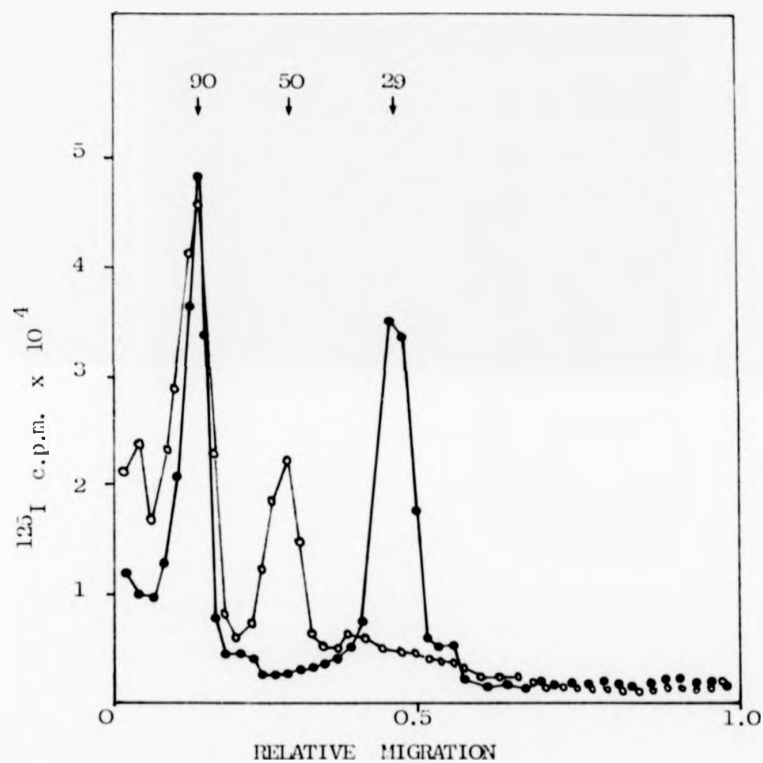


Figure 23

Identification of glycopeptides in the light bands of protein (density 1.23 g cm^{-3}) in sucrose gradient containing IBV solubilised in 2% Triton X-100. Profile of radioactivity obtained after incubating ^{125}I -concanavalin A with sliced gels containing protein solubilised in 2% SDS:

- in the presence of 5% 2-mercaptoethanol
- in the absence of 2-mercaptoethanol.

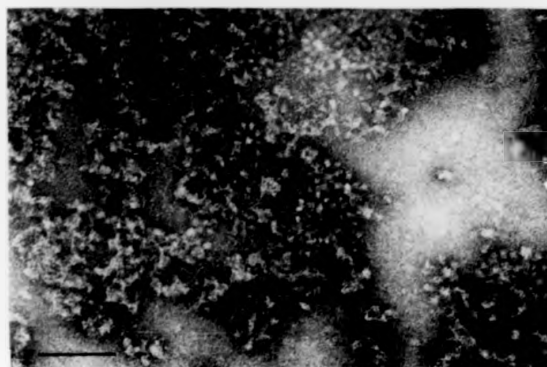


Figure 24

Electron micrograph of material banded at a density of 1.23 g cm^{-3} in sucrose after disruption in Triton X-100. Bar represents 100 nm.

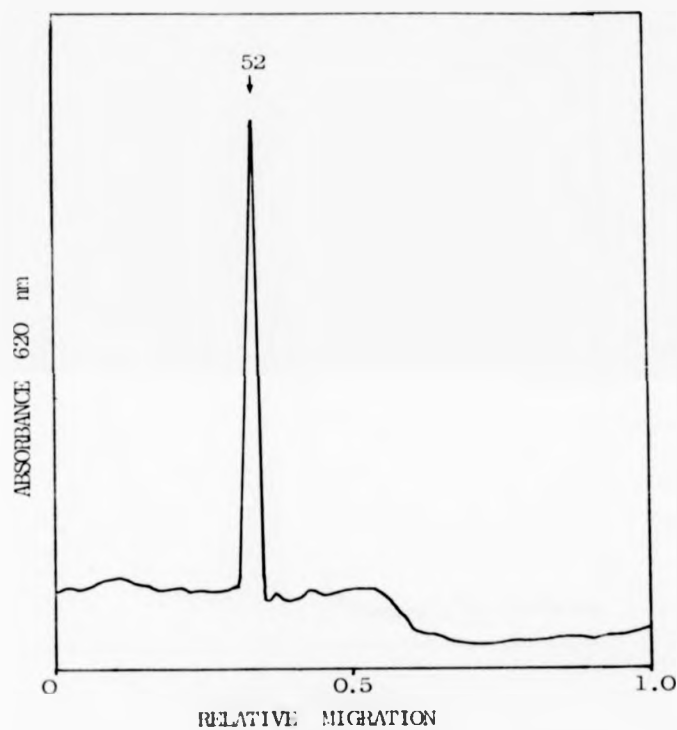


Figure 25

SDS-polyacrylamide gel of material pelleted to the bottom of sucrose gradient after disruption in Triton X-100. The band was dialysed against GITE buffer for 6 hours then disrupted in 2% SDS and 5% 2-mercaptoethanol prior to SDS-PAGE. Protein bands were stained with Coomassie Blue.

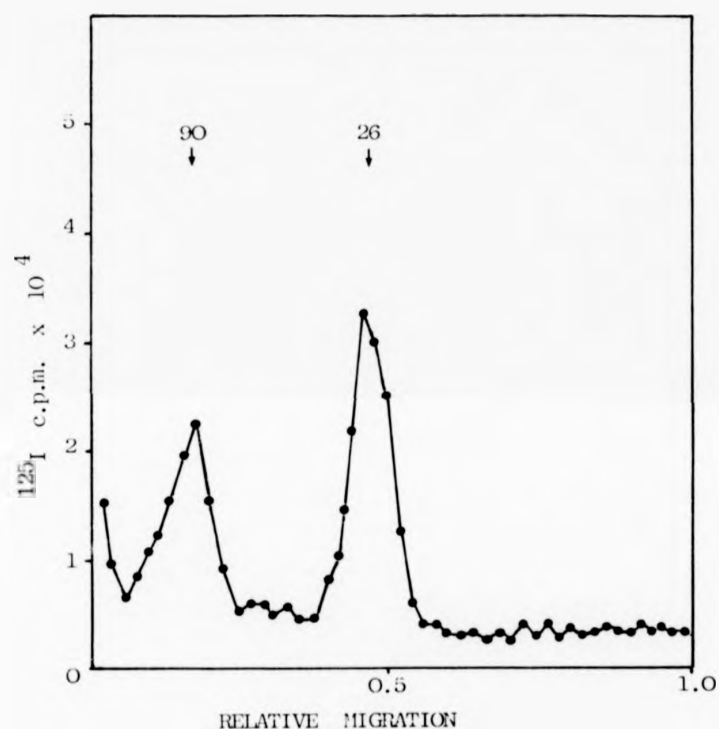


Figure 26

Identification of glycopeptides in SDS-polyacrylamide gels of material pelleted to the bottom of sucrose gradient after solubilisation of IBV in Triton X-100. Profile of radioactivity obtained after incubating ^{125}I -concanavalin A with sliced 10% polyacrylamide gels of material from the pellet. Samples for SDS-PAGE had been solubilised in 2% SDS and 5% 2-mercaptoethanol.

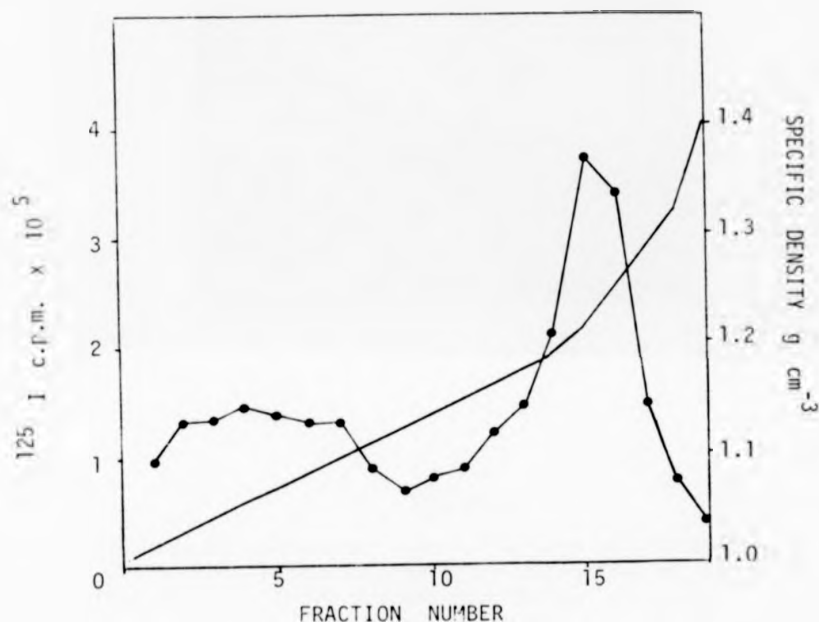


Figure 27

Sedimentation profile in Dextranide of purified ^{125}I -IPV and unlabelled IPV which had been solubilised in Triton X-100 and then centrifuged for 24 hours at 150,000 g in a Sorvall AM-650 rotor. Two bands of protein were obtained; one at a density of 1.22 g cm^{-3} and another at the bottom of the gradient. Only one band of radioactivity was obtained at a density of 1.22 g cm^{-3} .

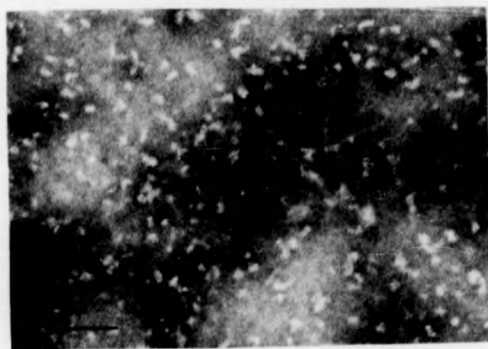


Figure 23

Electron micrograph of material banded at a density of 1.22 g cm^{-3} in Metrizamide after disruption of IBV in Triton X-100. Bar represents 100 nm.

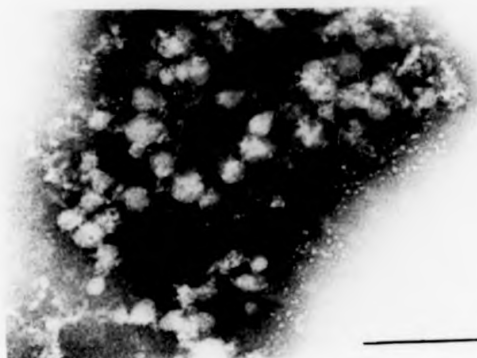


Figure 29

Electron micrograph of material with a density greater than 1.32 g cm^{-3} in Metrizamide after disruption of IBV in Triton X-100. Bar represents 100 nm.

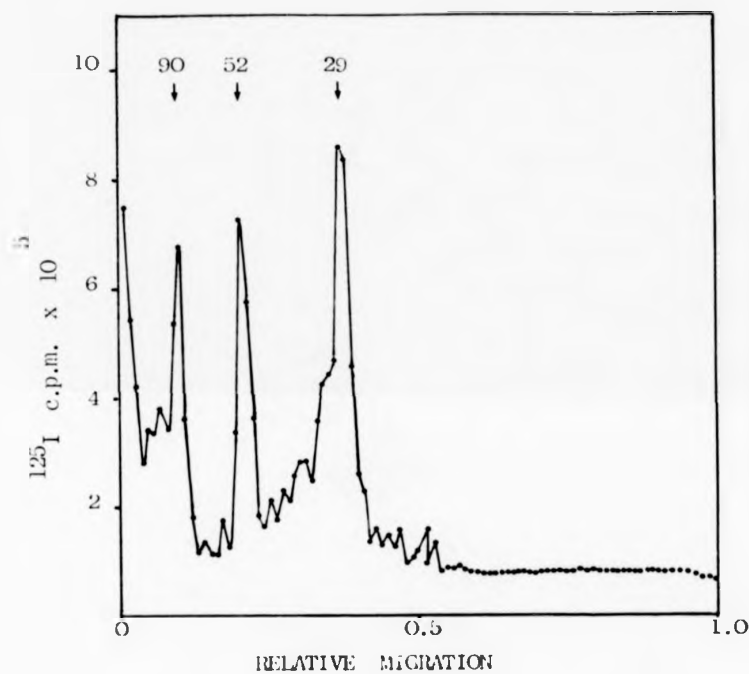


Figure 30

SDS polyacrylamide disc gel electrophoresis in 10% gels of radiolabelled material at a density of 1.22 g cm^{-3} in Metrizamide gradients containing ^{125}I -IBV solubilised in Triton X-100. Samples for SDS-PAGE were disrupted in 2% SDS and 5% 2-mercaptoethanol.

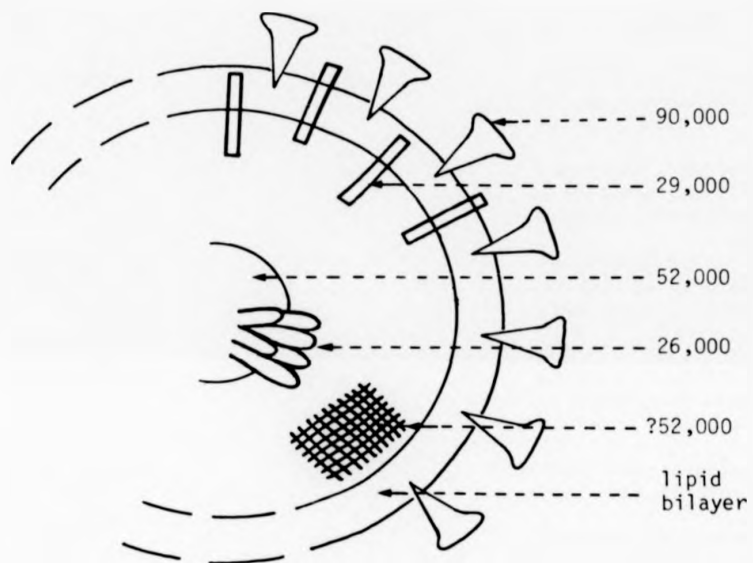


Figure 31

Model for structure of IBV.

TABLE 1

RECOGNISED CORONAVIRUSES OF MAN AND ANIMALS

<u>Species</u>	<u>Animal Host</u>	<u>Disease</u>
Infectious bronchitis IBV	chicken	Respiratory, ovarian damage mortality in young chicks
Mouse hepatitis MHV	mouse	hepatitis, encephalitis, diarrhoea subclinical, endemic infections
Transmissible gastro- enteritis TGEV	pig	gastroenteritis, high mortality in piglets less than 2 weeks
Haemagglutinating encephalomyelitis HEV	pig	encephalomyelitis in piglets high mortality in less than 2 weeks
Neonatal calf diarrhoea coronavirus NCDV	calf	diarrhoea in calves, may be fatal in neonates
Human HCV enteric HECV	man	respiratory infection, associated with gastro- enteritis
Rat coronavirus RCV	rat	pneumonitis, high mortality in rats less than 48 hours old
Sialodacryoadenitis SDAV	rat	acute disease of maxillary and lacrimal glands
Coronavirus Enteritis of turkeys CET	turkey	enteritis, bluecombe, may be high mortality
Canine coronavirus CCV	dog	gastroenteritis, severe in puppies
Feline infectious peritonitis FIP	cat	peritonitis

TABLE 2

POLYPEPTIDES OF CORONAVIRUSES OTHER THAN IBV

HCV OC43	HCV 229E	TGEV	HEV	NCDCV	MHV A59	MHV JHM
Molecular Weight x 10 ⁻³						
		200 G *				
191 G	196 G					
			180 G		180 G	
165 G	165 G					170 G
			125 G			125 G
				110 G		
104 G	105 G	105	100 G	100 G		97 G
					90 G	
		80		82		
				70		
60 G	65 G					60
			56			
47	47	50		53 G	50	
				45 G		
				36		
30	31 G	30 G				
		28 G	26 G			24 G
					23 G	22
15 G	16 G					

* G represents glycosylation

TABLE 3

POLYPEPTIDES OF IBV

Bingham 1975	Collins et al 1976	Alexander Collins 1977	MacNaughton Madge 1977
Molecular weight x 10 ⁻³			
180 G*			
	150		
		135	
130 G	130		130 G
106		102	105 G
	94 G	92	97
83 G		86 G	
	78 G	78	81 G
		74	74 G
70		68 G	
		63 G	
58			
53	52 G	52	51
49	45	47	
		39	
37 G	34	36 G	33
32	32 G	32	
29		27	
24			
16		18	
14		15	

* G represents glycosylation

TABLE 4

MOLECULAR WEIGHTS OF CORONAVIRUS RNA

<u>Species</u>	<u>Molecular weight x 10⁶</u>	<u>Reference</u>
IBV	0.5 - 3.0 (heterogeneous)	Tannock, 1973
	9.0	Watkins et al, 1975
	5.5 - 5.7	Schochetman et al, 1977
	8.0	Lomniczi and Kennedy, 1977
	5.8	MacNaughton and Madge, 1977
	5.6	Simpson et al, 1978
HCV 229E	5.8	Macnaughton and Madge, 1978
HCV OC43	6.1	Tannock and Hierholzer, 1977
MHV	5.4 - 6.5	Wege et al, 1978
	5.4	Lai and Stohlman, 1978

TABLE 5

Purification of IBV

	Protein mg/ml	Infectivity EID ₅₀ /ml	Volume ml	Purification ¹ factor
ALLANTOIC FLUID	3.0	10 ^{8.0}	2400	1
PEG	2.7	10 ^{10.5}	12	330
MET ₁	0.3	10 ^{10.5}	6	3300
MET ₂	0.2	10 ^{10.0}	3	1670

1. Purification factor is calculated by estimating total infectivity/mg protein at each stage of purification and dividing by the estimate obtained for allantoic fluid.