Haemagglutination by Avian Infectious Bronchitis Virus – a Coronavirus

By R. W. BINGHAM, M. HILARY MADGE AND D. A. J. TYRRELL

Division of Communicable Diseases, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, U.K.

(Accepted 5 May 1975)

SUMMARY

The haemagglutinating ability of three strains of IBV was investigated. It was shown that whereas strain Beaudette had no detectable haemagglutinin, both Connecticut and Massachusetts agglutinated red cells of various species. The haemagglutinin of Connecticut was detectable after sucrose gradient purification whereas that of Massachusetts required both the purification step and incubation with the enzyme phospholipase C to reveal it. The agglutination could be inhibited by specific antisera. Some studies on the nature of the red cell receptor, and the possible presence of a receptor destroying enzyme, are reported.

INTRODUCTION

Coronaviruses are grouped together on the grounds of their morphology, ether lability and apparent RNA content (Tyrrell *et al.* 1968). Otherwise they are rather non-uniform and so far only haemagglutinating encephalitis virus of pigs (HEV) and certain strains of human coronavirus, namely OC38 and OC43, have been shown to haemagglutinate. Both agglutinate red cells of several species (McIntosh, 1974). There have also been reports that avian infectious bronchitis virus (IBV) agglutinated chicken red cells, although only after the virus preparation had been pre-treated with trypsin (Corbo & Cunningham, 1959) – this agglutinin could apparently be separated from the virus particle (Biswal, Nazerian & Cunningham, 1966) and was not specifically inhibited by immune serum. It was observed only with the Massachusetts strain and others have had difficulty in repeating the observations.

In this paper we describe haemagglutination which is apparently due to the IBV particle.

METHODS

Virus strains and purification. The virus strains used, their growth and purification have been described previously (Bingham, 1975).

Antisera. Chicken immune sera against IBV-Massachusetts and IBV-Connecticut were supplied by Mr T. Hall, Wellcome Research Laboratories, Beckenham, Kent. Control chicken non-immune serum was collected from specific pathogen free chickens (Wickham Laboratories, Wickham, Hampshire). Other antisera and immune ascitic fluids were previously prepared and described by Bradburne (1970).

Red blood cells. Cells were collected in sterile anticoagulant (Heparin, 50 international units (i.u.)/ml for the chicken cells, Alsever's solution for the other species) and immediately washed by sedimentation and resuspension $(3 \times)$ in Dulbecco's phosphate buffered saline

382 R. W. BINGHAM, M. H. MADGE AND D. A. J. TYRRELL

'A' (PBSA; Oxoid Ltd) and either used immediately or stored at 4 °C as a 10 % suspension in dextrose gelatin-veronal solution (Clark & Casals, 1958). Cells were suspended in PBSA for use, chicken erythrocytes at 0.5 % and the other species at 1 %. For some experiments the pH of the suspension was altered by the use of phosphate buffers made in the manner described by Sørensen (1912), to which 0.4 % sodium chloride had been added to maintain tonicity.

Enzymic and chemical treatments of virus and erythrocytes. Trypsin (EC 3.4.21.4, Difco Laboratories Ltd) was used to treat purified virus in the manner described by Corbo & Cunningham (1959). Trypsin treatment of red blood cells was by the method of Buckland & Tyrrell (1963). Bromelain (EC 3.4.22.4, Sigma Chemical Co.) treatment of virus was by the method of Compans *et al.* (1970). Bromelain treatment of chicken red cells was accomplished by incubation under similar conditions, with the exception that the cells were suspended in PBSA.

Neuraminidase (EC 3.2.1.18, Behringwerke, 500 units/ml) was used to treat virus by the method of Witter *et al.* (1973) or to pre-treat chicken erythrocytes by incubation under similar conditions (without phospholipase), except that the enzyme was at 25 units/ml final concentration. Phospholipase C (EC 3.1.4.3, type I, from *Clostridium welchii*, 5 units/mg protein, and type III from *Bacillus cereus*, 67 units/mg), phospholipase A₂ (EC 3.1.1.4, from bee venom, 1550 units/mg) and phospholipase D (EC 3.1.4.4, type I, from cabbage, 19 units/mg) were obtained from Sigma Chemical Co., and used to treat virus in the manner described by Witter *et al.* (1973), at a final concentration of 0.25 units/ml. Periodate treatment of erythrocytes was carried out by the method (*b*) described by Buckland & Tyrrell (1963). After treatment of red cells by any method, they were washed by sedimentation and resuspension $(3 \times)$ in PBSA.

Haemagglutination test. Tests were carried out in Microtiter 'V' plates using the Takatsy microtitrator system (Cooke Engineering Co., Alexandria, Virginia). Virus was diluted in 0.025 ml volumes of PBSA and an equal volume of erythrocyte suspension was added to each cup. The procedure was routinely carried out at 4 °C except where otherwise indicated in Results. Patterns of agglutination were observed at the intervals specified in Results. Titres (H.A.U.) were recorded as the reciprocal of the highest antigen dilution (in 0.025 ml) causing a detectable haemagglutination. In the experiments in which pH was varied, the appropriate phosphate buffer (described above) was used for the dilutions.

Haemagglutination inhibition test. Sera were inactivated by heating at 56 °C for 30 min and serially diluted in the cups of a Microtiter plate in 0.025 ml PBSA as above. 0.025 ml of the antigen, adjusted to 4 H.A.U., was added to each well and the plate allowed to stand at 4 °C for 1 h before the addition of the red cells (0.025 ml). Haemagglutination inhibition titres (H.A.I.U.) were recorded as the reciprocal of the highest serum dilution (in 0.025 ml) causing a detectable inhibition of the agglutination.

Particle counts of virus. Preparations of virus were mixed with an equal volume of a standard suspension of latex particles and the mixture negatively stained with sodium phosphotungstate and examined by electron microscopy by Mr D. Gunner of this Institute. The concentration of virus particles was calculated from the ratio of virus to latex particles observed.

RESULTS

Haemagglutination was first observed when a fraction of a sucrose gradient which was rich in virus was tested with chicken red cells. The pattern was clearly seen on initial settling but seemed to be unstable. Accordingly, the conditions necessary for haemagglutination

		fractions of virus subjected to treatments specified					
Strain	Untreated [†]	Phospholipase C (I)	Phospholipase ospholipase C (I) C (I) + Neuraminidase		Trypsin		
Connecticut	384	384	o (<4)	0 (<4)	N.D.‡		
Massachusetts	0 (< 4)	32	6	0 (< 4)	0 (< 4)		
Beaudette	0	o (< 4)	o (<4)	0 (< 4)	N.D.		
Control§	0 (< 4)	0 (< 4)	I 2	2	0 (< 4)		

Table 1.	Haemagglutination	by three	strains a	of IBV	treated i	in various	ways

Haemagglutination titres* (H.A.U./0.025 ml) for sucrose gradient

* Corrected for the ‡ dilution resulting from the enzyme treatment. Chicken red cells were used.

† Diluted $\frac{1}{4}$ in PBSA – except for Beaudette (undiluted).

‡ Not done.

§ Control consisted of 40 % sucrose in PBSA incubated with the appropriate enzyme and serially diluted in same manner as virus. Each of the virus fractions contained 5 to 10 mg protein/ml, estimated by E_{280}/E_{260} measurements.

were systematically investigated. We were particularly interested in the possibility of unmasking haemagglutinin by enzymic treatment of the particles.

Virus preparations and pre-treatments

Three strains were tested in the form of sucrose gradient fractions. We used trypsin as described by Corbo & Cunningham (1959) and also phospholipase C and neuraminidase which has been used to reveal the haemagglutinin of a mouse oncornavirus (Schäfer & Szántó, 1969; Witter *et al.* 1973) and also of avian myeloblastosis virus (Szántó, 1971). As can be seen in Table I the only treatment which increased the haemagglutination titre was phospholipase C on its own and it had this effect only on IBV-Massachusetts. Trypsin and neuraminidase seemed to inactivate the haemagglutinin.

From these results it was decided to concentrate on the two strains, Connecticut and Massachusetts, which had haemagglutinin, and to study the differences between the two. Accordingly, fresh harvests of egg-grown virus of both strains were purified on sucrose gradients and individual fractions of the gradients examined for agglutinating activity; these results are given in Fig. 1. The two densities (1.18 and 1.22) at which the virus bands (Bingham, 1975) are reflected in the distribution of haemagglutinin in the gradient. It can also be seen that the phospholipase treatment revealed haemagglutinin in fractions that had previously no agglutinin, yet had little effect on the fractions which had the highest titre before treatment. This may mean that there are different populations of virus particles at the different densities which respond in dissimilar ways to the treatment, or that some of the agglutination is due to subviral pieces. We also found that the HA titres diminished after storage of the gradient fractions at -70 °C, and the high titres in the freshly harvested, unfrozen, gradient fractions could not be achieved in the later experiments using pooled material which had been kept frozen for varying periods. Continuing loss of activity on storage meant that titres achieved in one experiment could not be reproduced if the same sample was titrated at a later date. The peak agglutinating fractions (10 to 13 and 17 to 21, both strains) were pooled to give light and heavy fractions for each strain – designated L and H respectively. These four fractions or similar fractions prepared in the same manner, were then used for the subsequent studies on the nature of the agglutination reaction.

Following up these observations it was noted that bromelain treatment also abolishes



Fig. 1. Haemagglutination in 1 ml fractions from sucrose gradients after sedimentation of IBV (a) Massachusetts, (b) Connecticut: $\triangle --- \triangle$, density of sucrose in fractions, estimated by refractometry; $\blacksquare --- \blacksquare$, particle count (log₁₀ virus particles/ml); $\blacksquare --- \blacksquare$, HA titres without any treatment; $\bigcirc --- \bigcirc$, HA titres with phospholipase C (type I) treatment.

haemagglutinin (Table 2), and this enzyme is known to remove the club-shaped projections from the surface of coronaviruses (Hierholzer *et al.* 1972; S. Patterson & R. W. Bingham, unpublished results). Electron microscopy by Dr J. D. Almeida of the strains of virus used here showed that Connecticut particles usually had a good array of projections, Beaudette usually had none, while Massachusetts had projections which were at times partly obscured by ill defined material, although it was purified by exactly the same procedures as the other viruses. The observation that the two of our viruses which haemagglutinated also had surface projections, suggested that this structural feature was concerned with the haemagglutination.

Agglutination of erythrocytes from various species

Although most of the experiments reported here were performed with chicken red cells, we were interested in the possibility that other types of cell would be suitable for the reaction. Table 3 lists the results obtained using a variety of red cell types. It may be seen that guineapig cells gave the highest titres and that human and dog cells were also suitable for use in

	Haemagglutinin titre after indicated treatment						
Strain (fraction)	Untreated	Phospholipase C (I)	Phospholipase C (I) + bromelain	Bromelain			
Massachusetts (L)	0	24	0	0			
Massachusetts (H)	0	16	0	0			
Connecticut (L)	64	192	0	0			
Connecticut (H)	0	64	0	0			
Control	0	3	3	0			

Table 2. Effect of bromelain treatment on haemagglutinin titres*

* Units, dilutions, controls and cells as stated for Table 1.

Phospho	olipase C (I)	reated	Untreated				
Mass. (L)	Conn. (L)	Control	Mass. (L)	Conn. (L)	Control		
64	384	3	I	384	0		
128	512	6	16	> 2048	0		
32	256	ο	I	512	0		
0	64	0	2	2	0		
32	128	3	2	64	0		
128	256	3	32	128	0		
16	0	4	16	8	3		
	Phosphc Mass. (L) 64 128 32 0 32 128 16	Phospholipase C (I) 1 Mass. (L) Conn. (L) 64 384 128 512 32 256 0 64 32 128 128 256 16 0	Phospholipase C (I) treated Mass. (L) Conn. (L) Control 64 384 3 128 512 6 32 256 0 0 64 0 32 128 3 128 256 3 128 256 3 128 256 3 128 256 3 16 0 4	Phospholipase C (I) treated Mass. (L) Conn. (L) Control Mass. (L) 64 384 3 I 128 512 6 16 32 256 0 I 0 64 0 2 32 128 3 2 128 256 3 32 128 256 3 32 16 0 4 16	$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$		

Table 3. Haemagglutination titres with cells of various species*

Antigen

* Units, dilutions, and controls as stated for Table 1.

the test. Agglutination with sheep and rabbit cells was rather poor, and there was evidence of a difference in affinity for the two strains of virus. All the cells used for this test were freshly taken from the animals, and it is pertinent to note that we observed that chicken cells had to be used within 4 days of collection. Cells kept longer than that period agglutinated poorly or not at all.

Suspending media and other conditions

In early experiments it appeared that the solutions in which the test was done were not critically important. The titres were unchanged when the dilutions were made in PBSA, complete PBS, saline, or PBSA + 0.2 % borine serum albumin. There was no significant difference in titres for tests performed at pH 6.0, 6.35, 7.0, 7.4, or 8.2. The temperature at which the cells settled was important. The titres were rather higher at 4 °C than at room temperature and lower at 37 °C. Furthermore, as shown in Table 4, as the test was observed over a period the titres declined more rapidly at higher temperatures. In addition prozones were noted with both viruses (both light and heavy fractions) with and without phospholipase treatment. As can be seen in Fig. 2(a) by about $1\frac{1}{2}$ h the haemagglutination pattern has disappeared in the cups containing the highest concentration of virus in which agglutination patterns were at first evident. This is not due to 'slipping' of the cell layer since, if the cells are resuspended and allowed to settle again, haemagglutination does not recur in the cups in the prozone. It was thought that this might be a result of complete covering of the receptors of all cells, which would not allow virus particles to form bridges. However it was found that supernatant fluids from the disagglutinated cells contained haemagglutinin

				Observed	end-poin	t (H.A.U.)			
Time reading		Connecticut		Massachusetts		etts	Control*		
taken†	΄4 ℃	21 °C	37 °C	΄₄ °C	21 °C	37 °C	4 °C	21 °C	37 °C
20 min	64	32	32	48	32	16	I 2	32	16
35 min	64	24	32	32	16	16	8	16	16
50 min	64	24	32	32	16	8	8	3	6
90 min	64	24	32	32	4	8	2	3	4
120 min	64	24	32	32	4	4	0	3	4
60 h	64	16	‡	2	4	‡	0	3	‡

Table 4. Stability of haemagglutination pattern at different temperatures for two strains of IBV treated with phospholipase C (I)

* Control as described in Table 1. With PBSA alone, complete settling of cells had occurred by 35 min at all three temperatures.

† Readings of H.A.U. observed at stated times after addition of erythrocytes (chicken).

‡ Cups dried out.

to the same titre as had originally been added. If a freshly made mixture of red cells and virus of known titre was centrifuged and the supernatant fluid titrated, the amount of agglutinin was found to be reduced. On incubation of parallel mixtures at 37 °C and subsequent titration of the supernatant fluid, the titres increased to the original input level. With strain Connecticut it was observed that the prozone formed at 4 °C within a few hours, but then did not enlarge even if the plate was kept for several days at 4 °C. However on warming the plate to 37 °C, all the cups that still had agglutination settled out within 60 min. The patterns produced by Massachusetts were never really stable and tended to weaken and disappear in a matter of hours at 4 °C. This suggested that some process was causing the virus to elute from the red cells, and that there was a considerable difference in the rate of the effect in different strains.

Inhibition of haemagglutination by specific antisera

In a search for further evidence that the agglutination was virus-specific we performed haemagglutination inhibition tests. Specific neutralizing antiserum against each strain was found to inhibit the agglutination of both strains (Table 5) whereas the control serum had very little inhibitory effect. We obtained similar results when the inhibition test was carried out using human red cells. This differs from the results obtained in neutralization tests, where the anti-Connecticut serum neutralizes both strains equally (Neutralization Index titre with 1/5 diluted sera (N.I.) > 4 for both strains), but the anti-Massachusetts serum is specific for Massachusetts only (N.I. 2.5 for Massachusetts only). In view of this, tests were also performed with antisera and immune ascitic fluids against other coronaviruses to which IBV is not related by other tests (Bradburne, 1970). Non-specific inhibition was very high (up to 2048 units) with all the rabbit sera and mouse ascitic fluids. As a result, specific inhibition was not detectable with rabbit sera prepared against human coronavirus (HCV) 229E, or with mouse ascitic fluids prepared against HCV 229E, HCV OC43 or mouse hepatitis virus. Fig. 2(b) illustrates inhibition of haemagglutination by specific chicken antisera.

Non-specific inhibition of haemagglutination

As haemagglutination is only apparent after purification on sucrose gradients, it was thought that receptor-like substances might be found in eggs as 'non-specific' (i.e. nonantibody) inhibitors of haemagglutination. Such inhibition had already been shown in



Fig. 2. (a) Haemagglutination by IBV: A, PBSA control, chicken cells; B, Connecticut (L), diluted 1:4, no treatment, chicken cells; C, Connecticut (L), phospholipase C treated, chicken cells; D, Massachusetts (L), diluted 1:4, no treatment, chicken cells; E, Massachusetts (L), phospholipase C treated, chicken cells; F, as C, guinea-pig cells; G, as C, rabbit cells; H, sucrose/phospholipase C control, chicken cells. (b) Haemagglutination inhibition by chicken sera: A, anti-Connecticut serum ν . Connecticut (L); B, anti-Connecticut serum ν . Massachusetts (L); C, anti-Massachusetts serum ν . Connecticut (L); D, anti-Massachusetts serum ν . Massachusetts (L); E, specific pathogen-free chicken serum ν . Connecticut (L); F, specific pathogen free chicken serum ν . Massachusetts (L); G, PBSA control. All rows with chicken cells. Experimental procedures were as described in Methods.

Table 5. Inhibition of haemagglutination by specific antisera

Inhibition titres (H.A.I.U./0.025 ml) for indicated sera*

	Chicken an	Specific pathogen		
Antigen†	Connecticut	Massachusetts	free chicken serum	
Connecticut (L)	12	96	4	
Connecticut (H)	48	192	4	
Massachusetts (L)	24	64	o (<4)	
Massachusetts (H)	96	96	12	

* All sera were diluted $\frac{1}{4}$ in PBSA before use, quoted titres are corrected for this dilution.

† Antigen was treated with phospholipase C (I) and adjusted to 4 H.A.U./0.025 ml before use.

Strain+	Method of treatment of chicken erythrocytes						
(fraction)	Untreated	Trypsin	Bromelain	Periodate	Neuraminidase		
Connecticut (L)	128	32	12	128	12		
Connecticut (H)	128	32	0 (< 4)	I 28	o (<4)		
Massachusetts (L)	48	4	o (< 4)	72	0 (< 4)		
Massachusetts (H)	48	3	0 (< 4)	I 28	0 (< 4)		
Control	8	6	I 2	8	12		

 Table 6. Haemagglutination with treated erythrocytes*

* Units, dilutions, and controls as for Table 1.

† Phospholipase C (I) treated.

normal rabbit serum and mouse ascitic fluid, so further tests were done with allantoic fluid (clarified by sedimentation) and 10 % extracts in PBSA of yolk or chorioallantoic membrane (as homogenate) from specific pathogen-free eggs. The inhibition tests were performed in identical fashion to those with antisera except that the inhibitors were not pre-heated. Each of these substances caused some inhibition, the allantoic fluid inhibiting to a titre of 3 H.A.I.U./0.025 ml and the yolk and chorioallantoic membrane extracts both caused partial inhibition of agglutination to a titre of 32 H.A.I.U./0.025 ml. Although the inhibition titre of the allantoic fluid is low, it is sufficient to account for the failure to detect agglutination using fluid from infected eggs without prior purification of the virus.

Red cell receptors

It has already been indicated that virus haemagglutinin adsorbs to red cells and then elutes, and the next experiments were designed to investigate the chemical nature of the sites with which the virus can react. Red cells were treated with relatively specific reagents known to affect receptors for other viruses. In general a concentration was used which was as high as could be conveniently reached without causing lysis, non-specific agglutination or other adverse effect. The results of one experiment are shown in Table 6 – similar results were obtained for a repeat experiment. This shows that periodate had no effect, trypsin had a slight effect on the agglutination with Connecticut and abolished the agglutination with Massachusetts, and neuraminidase and bromelain both abolished agglutination completely. However it was observed that with periodate, although the haemagglutination titre was unaltered, the formation of the prozone was prevented and in the case of Massachusetts (normally unstable if kept overnight) the titre remained unaltered after 24 h.

These results suggest that the receptor is a glycopeptide which is totally removed from the red cell by bromelain, but perhaps only partially cleaved by trypsin, leaving a shorter glycosylated portion still able to react with the virus agglutinin. The carbohydrate portions would also appear to carry terminal *N*-acetyl neuraminic acid molecules essential to the reaction. Periodate oxidation seems to modify the receptor in such a way that although agglutination is not affected, the elution mechanism is prevented.

Pre-treatment of virus with different phospholipases

In an attempt to gain further information on the unmasking phenomenon caused by the phospholipase treatment, virus was treated with some other types of phospholipase. Samples of virus were incubated under identical conditions with the same activities of phospholipase A_2 , phospholipase C (type I), phospholipase C (type III) or phospholipase D and the agglutinating ability after treatment compared with untreated virus. In the case of Massachusetts

Haemagglutination by IBV

strain virus only phospholipase C (type I) revealed the haemagglutinin. With the Connecticut strain only phospholipase C (type I) exerted the usual enhancing effect on the HA titres – the other phospholipases neither increased nor decreased the titres.

DISCUSSION

It will no doubt be asked why haemagglutination by IBV has not been described before, since it seems now to be easy to obtain. There are probably several parts to the answer. One is that there are large differences between strains in their agglutinating activity; in addition even the most active strain must be concentrated and purified before any effect on red cells can be observed. Also, the variation in HA titre caused by the age of the erythrocyte preparation, along with the diminishing titre on storage, means that unless freshly purified virus is used with freshly collected red cells, the effect may not be detected.

Detailed comparisons with the haemagglutinins of other coronaviruses i.e. OC43 and HEV show some similarities. For instance haemagglutination occurs with cells from a wide range of species, although there is no relationship between the types of cell with which agglutination will and will not occur for the three different types of virus (McIntosh, 1974). It seems to be mediated by the virus particle, and is abolished in OC43, as in IBV, by removing the spikes with bromelain.

Haemagglutination inhibition may be of practical value in the study of disease since it might be used in diagnosis and in detecting response to vaccines. It remains to be seen whether it is as convenient or useful as other tests already in use for IBV. Since it is more broad reacting antigenically than neutralization, it may not be suitable in some instances for detecting specific immunity to particular strains.

It will be of interest to study further the exact nature of the haemagglutinating portion of the virus envelope. In the human virus, OC43, it has been suggested that the haemagglutinating activity is carried by one or both of the glycopeptides of the projections (Hierholzer *et al.* 1972). However IBV seems to be somewhat different in its peptide composition (Bingham, 1975) and more work is needed to relate peptides to haemagglutinin.

The formation of prozones, and the instability of the agglutination on warming suggests that there might be a viral enzyme, such as neuraminidase, responsible for uncoupling the virus from the cell-surface receptor. This possibility is currently being investigated. So far, the presence of neuraminidase has not been described for any purified coronavirus, but this does not rule out the possibility of a different type of receptor-destroying enzyme such as that described by Kendal (1974) for influenza C virus. Since there is a strain difference for this phenomenon, and it is independent of phospholipase incubation, it is not an artifact of the pre-treatment. Elution of the human coronavirus strains OC43 and OC38 from human 'O' and vervet erythrocytes, at room temperature, has been demonstrated by Kaye & Dowdle (1969). However, there was no apparent loss of cell receptors, nor did elution occur from chicken, rat or mouse cells. There are no reports of elution of HEV from red cells.

It is still not clear how phospholipase treatment either enhances or reveals the haemagglutinin. Electron microscopic examination of phospholipase C (type I) treated particles shows no detectable difference n the morphology of the virus (S. Patterson & R. W. Bingham, unpublished results). However it may need only a slight change in the physical character of the virus envelope lipid to cause sufficient alteration in the position of a polypeptide in that membrane to enable it to assume a suitable configuration for agglutination. Hydrolysis of the phosphatidyl choline in a typical lipid bilayer membrane is likely to cause an increase in membrane fluidity without necessarily resulting in lysis (S. Johnson, personal

R. W. BINGHAM, M. H. MADGE AND D. A. J. TYRRELL

390

communication). Analysis by thin layer chromatography of extracts of lipids from samples of the virus incubated with each of the different phospholipases showed that the phospholipase C (type I) caused total hydrolysis of the virus phosphatidyl choline, whereas the type III enzyme, phospholipase A_2 and phospholipase D, had little or no apparent effect on the phosphatidyl choline (R. W. Bingham & S. Johnson, unpublished results). Commercial preparations of these enzymes are not necessarily pure; indeed the phospholipase C (type I) used here contained low levels of neuraminidase activity (M. H. Madge & R. W. Bingham, unpublished results) and may also contain other enzymic activities (Diner, 1970; Zwaal *et al.* 1971). Therefore some other uncharacterized activity in the enzyme preparation may be responsible for the effects on the virus haemagglutinin.

Further characterization of these effects, and application of these techniques to other apparently non-haemagglutinating coronaviruses may prove to be rewarding.

We wish to thank Mrs S. MacLauchlan for technical assistance, Dr J. D. Almeida, Dr S. Patterson and Mr D. Gunner for performing the electron microscopy, and Dr S. Johnson for advice and assistance with the chromatography of the lipids. We are indebted to Mr T. Hall for the specific chicken antisera.

REFERENCES

- BINGHAM, R. W. (1975). The polypeptide composition of avian infectious bronchitis virus (in the press).
- BISWAL, N., NAZERIAN, K. & CUNNINGHAM, C. H. (1966). A haemagglutinating fraction of infectious bronchitis virus. *American Journal of Veterinary Research* 27, 1157–1167.
- BRADBURNE, A. F. (1970). Antigenic relationships amongst coronaviruses. Archiv für die gesamte Virusforschung 31, 352-364.
- BUCKLAND, F. E. & TYRRELL, D. A. J. (1963). A comparative study of virus haemagglutinins. The stability of haemagglutinins and red cell receptors to certain physical and chemical treatments. *Journal of General Microbiology* 32, 241-253.
- CLARKE, D. H. & CASALS, J. (1958). Techniques for haemagglutination and haemagglutination inhibition with arthropod-borne viruses. *American Journal of Tropical Medicine and Hygiene* 7, 561–573.
- COMPANS, R. W., KLENK, H. D., CALIGUIRI, L. A. & CHOPPIN, P. W. (1970). Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycopeptides. *Virology* **42**, 880–889.
- CORBO, L. J. & CUNNINGHAM, C. H. (1959). Haemagglutination by trypsin-modified infectious bronchitis virus. American Journal of Veterinary Research 20, 876–883.
- DINER, B. A. (1970). Purification and properties of *Clostridium welchii* phospholipase C. *Biochimica et Biophysica Acta* 198, 514–522.
- HIERHOLZER, J. C., PALMER, E. L., WHITFIELD, S. G., KAYE, H. S. & DOWDLE, W. R. (1972). Protein composition of coronavirus OC43. Virology 48, 516-527.
- KAYE, H. S. & DOWDLE, W. R. (1969). Some characteristics of haemagglutination of certain strains of 'IBVlike' virus. *The Journal of Infectious Diseases* 120, 576–581.
- KENDAL, A. P. (1974). A new receptor destroying enzyme in influenza C? Behring Institute Mitteilungen 55, 18-31.
- MCINTOSH, K. (1974). Coronaviruses; a comparative review. Current Topics in Microbiology and Immunology 63, 85–129.
- SCHÄFER, W. & SZÁNTÓ, J. (1969). Studies on mouse leukaemia viruses. II. Nachweis eines virusspezifischen Hämagglutinins. Zeitschrift für Naturforschung **B24**, 1324–1331.
- SØRENSEN, S. P. L. (1912). Uber die Messung und Bedeutung der Wasserstoffionenkonzentration bei biologischen Prozessen. Ergebnisse der Physiologie 12, 393-532.
- SZÁNTÓ, J. (1971). Haemagglutination reaction with avian myeloblastosis virus. Acta Virologica (Prague) 15, 245–248.
- TYRRELL, D. A. J., ALMEIDA, J. D., BERRY, D. M., CUNNINGHAM, C. H., HAMRE, D., HOFSTAD, M. S., MALLUCCI, L. & MCINTOSH, K. (1968). Coronaviruses. *Nature, London* 220, 650.
- WITTER, R., FRANK, H., MOENNIG, V., HUNSMANN, G., LANGE, J. & SCHÄFER, W. (1973). Properties of mouse leukaemia viruses. IV. Haemagglutination assay and characterisation of haemagglutinating surface components. *Virology* **54**, 330-345.
- ZWAAL, R. F. A., ROELOFSEN, B., COMFURIUS, P. & VAN DEENEN, L. L. M. (1971). Complete purification and some properties of phospholipase C from *Bacillus cereus*. *Biochimica et Biophysica Acta* 233, 474–479.

(Received 20 February 1975)