

Electron Microscope Observations on the Entry of Avian Infectious Bronchitis Virus into Susceptible Cells

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With 6 Figures

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Summary

Infectious bronchitis virus was observed to enter cells of chicken chorioallantoic membrane by viropexis. There was no support for the suggestion that entry took place by fusion of viral and plasma membranes. The results of electron microscopy showed that virus attachment occurred both at 4° and at 37° C. Viropexis was not observed until the preparations were warmed. Similar results were obtained using chicken kidney cells. Quantitative data obtained from a plaque counting system employing chicken kidney cells indicated that attachment was the same at both temperatures and that some virus particles were taken up at 4° C.

Virus uptake was triggered by attachment of the virus to the cell membrane and the subsequent process of virus entry visualised by E. M. appeared to proceed without the involvement of lysosomal enzymes. No intracellular virus was located by electron microscopy in warmed preparations when virus was treated with specific antiserum, either before or after adsorption to the cells.

Introduction

Avian infectious bronchitis virus (IBV) has been the subject of a number of ultrastructural investigations aimed at elucidating the mechanism by which this virus replicates (2, 14, 15, 19). However, no electron microscopic studies have paid attention to the mode of entry of IBV or other coronaviruses into susceptible cells.

According to a recent review (8), viropexis and fusion of the viral envelope with the cell membrane are the two main routes by which viruses gain access to the interior of cells. In order to establish the importance of these or other possible routes of entry by IBV, the early stages of infection have been monitored by electron microscopy, both in cells of the chorioallantoic membrane (CAM) and in

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chick kidney cells (CKC). The effect of antibody on the infectious process and the role of lysosomal enzymes in this system have been investigated, and some quantitative studies on the attachment and uptake of infectious virus into CKC were undertaken.

Materials and Methods

Virus Preparations

IBV-Beaudette was grown in eggs as described previously (4). The harvested allantoic fluid was clarified by sedimentation at $16,000 \times g$ for 15 minutes and the virus deposited at $75,000 \times g$ for 1 hour. The virus pellets were resuspended in some of the supernatant fluid to a final volume of $1/300$ th of the original. The whole procedure was carried out at 4°C and the resulting virus suspension was used for the adsorption experiments within 3 hours of being harvested. Heat inactivated virus was prepared by incubation of some of the suspension at 56°C for 30 minutes.

Chorioallantoic Membrane Preparations

The embryo and fluid were removed from 11 day incubated eggs and the membrane with its attached shell cut into small triangular pieces. The pieces were placed in the wells of a plastic tray (Linbro 96CV-TC, Biocult Laboratories Ltd.), overlaid with L-15 medium and the wells then sealed with paraffin film. Preparations were maintained at 4°C and immediately prior to the application of $200 \mu\text{l}$ of the virus suspension, excess medium was removed. After a one hour incubation at 4°C , some samples were fixed in buffered glutaraldehyde whilst the remainder were placed in a 37°C incubator and subsequently fixed at 5, 10, 20, 30 and 60 minute intervals. Control uninfected samples were processed simultaneously with virus infected preparations.

Ferritin

Equal volumes of ferritin (100 mg/ml) and the virus preparation were thoroughly mixed. Aliquots ($200 \mu\text{l}$) of this suspension were then applied to the chorioallantoic membranes and these were then processed as described above.

Antiserum Treatment

Chicken immune serum against IBV-Connecticut (3) was heated at 56°C for 30 minutes and mixed undiluted with an equal volume of the virus preparation. The mixture was incubated for 1 hour at 4°C and subsequently applied to chorioallantoic membranes. IBV-Beaudette is of the Massachusetts serotype, against which this antiserum had a Neutralisation Index titre (with $1/5$ diluted serum, N.I.) > 4 . Other membrane preparations were first subjected to incubation for 1 hour at 4°C with the virus preparation and then treated with $200 \mu\text{l}$ of the antiserum. These samples were maintained for a further hour at 4°C . Both antibody treated preparations were incubated for 1 hour or 17 hours at 37°C and then fixed in buffered glutaraldehyde. Control experiments were conducted in which the CAM-virus preparations were not subject to antibody treatment prior to the 17 hours 37°C incubation. The methodology of this series of experiments is summarised in Table 1.

Estimation of Adsorption and Uptake of Virus by Plaque Formation Assays

Replicate monolayers of chicken kidney cells were grown in 5 cm diameter vented plastic dishes (A/S Nunc, Denmark) as described previously (4) and inoculated with 0.5 ml of a suitable dilution of IBV that would give 20–30 plaques per plate under optimal conditions. The virus was diluted in maintenance medium consisting of L-15 with no serum, supplemented with 4 per cent tryptose phosphate broth, 100 units/ml penicillin and $100 \mu\text{g/ml}$ streptomycin. Virus was either inoculated directly or mixed with an equal volume of a $1/40$ dilution of the preheated antiserum described above and incubated at 4°C for 1 hour before addition to the plates. The antibody-treated or untreated virus was allowed to adsorb to the monolayer for 90 minutes at either 4° or 37°C as described in Table 2. At the end of the adsorption period, the plates that

had untreated virus were divided into three batches. To some, 0.5 ml of the antiserum was added and incubated for 30 minutes at the appropriate temperature, others were washed 3 times with maintenance medium (at the appropriate temperature) and the remainder overlaid directly without removal of the inoculum fluid. All the plates were overlaid with 3 ml of maintenance medium containing 1 per cent agarose (Indubiose A 37, L'Industrie Biologique Francaise S.A.) at 45° C, allowed to set, and incubated for 7 days at 37° C. The plaques produced were counted after neutral red staining of the cell sheet. Student's 't' test was used to calculate the statistical significance between groups.

Table 1. *Methodology of E.M. experiments employing immune serum against IBV*

Pre-treatment of virus preparation	Virus pre-incubated with anti-serum for 1 hour at 4° C	None	None	None
Treatment of CAM preparations after application of virus	Incubation for 1 hour at 37° C	Preparation incubated for 1 hour at 4° C; 200 µl antiserum added and incubation continued for further 1 hour at 4° C; finally incubated for 1 hour at 37° C	Preparation incubated for 1 hour at 4° C; 200 µl antiserum added and incubation continued for further 1 hour at 4° C; finally incubated for 17 hours at 37° C	Incubation for 17 hours at 37° C

In all experiments CAM preparations were precooled to 4° C before addition of the virus

Table 2. *Effect of temperature on attachment and entry of IBV into CKC*
Mean no. of plaques/plate \pm SD for inocula subjected to treatment specified*

Temperature during 90 minutes adsorption period	No antiserum, no washing	No antiserum, washed 3 \times at the end of adsorption	Antiserum added at the end of the adsorption period	Incubated with antiserum before adsorption
4° C	33.75 \pm 7.96	10.00 \pm 1.20	3.43 \pm 0.97	0.43 \pm 0.79
37° C	22.50 \pm 3.96	8.71 \pm 3.46	11.00 \pm 3.00	0.43 \pm 0.53

* Means of 7 or 8 replicate plates

Entry into Chicken Kidney Cells

Monolayers of CKC were prepared in wells of a Linbro tray and used for application of virus suspension. Preparations were fixed after incubation at 4° C for 1 hour and following warming at 37° C for 30 minutes, in the same fashion as the CAM preparations.

Electron Microscopy

After the experimental procedures described, the preparations were fixed in ice cold 3 per cent glutaraldehyde, buffered with 0.1 M cacodylate pH 7.4, for 1 hour. Thereafter the membranes were stripped from the shell and washed in several changes of 0.1 M cacodylate buffer, pH 7.4 containing 5 per cent (w/v) sucrose. This was followed by post fixation for 1 hour in 1 per cent OsO₄ in Millonig's buffer. After a brief wash in distilled water, the tissue was dehydrated in acetone and propylene oxide and embedded in Spurr resin (17). Embedding was carried out on flat trays on a cushion of infiltrated agar. Sections were stained in 5 per cent (w/v) uranyl acetate dissolved in 1 per cent acetic acid for 20 minutes followed by staining in Reynold's lead citrate

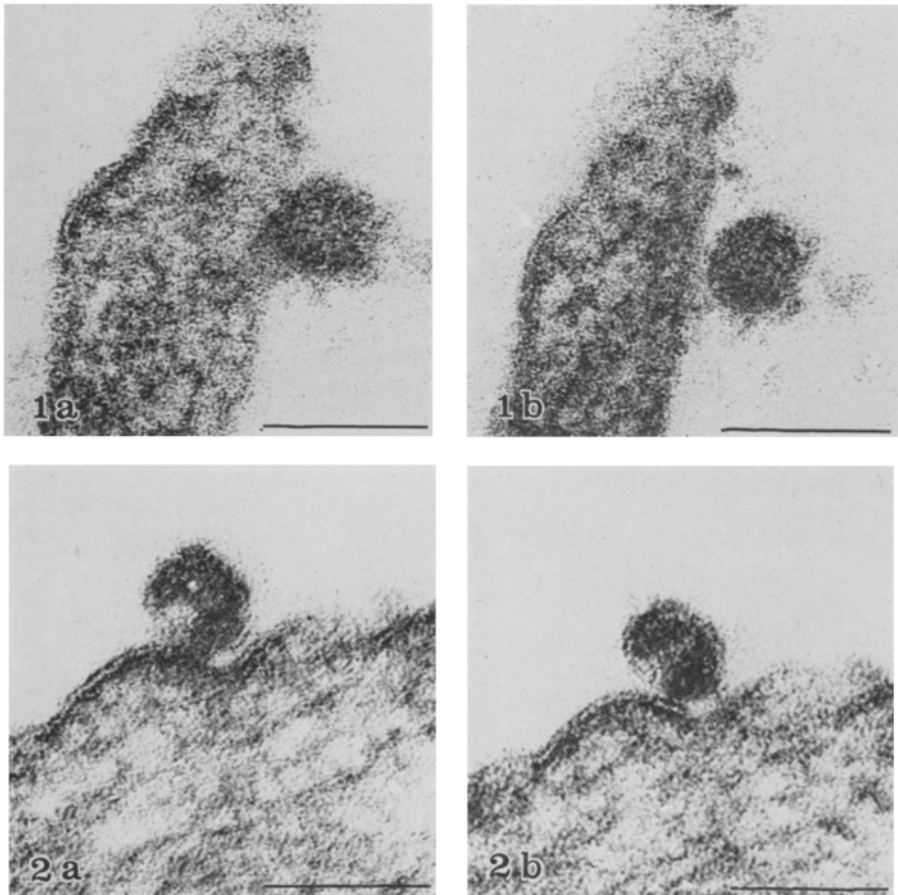
for 1 minute. They were coated with carbon and examined with a Philips EM 300 electron microscope which was equipped with a goniometer stage.

After glutaraldehyde fixation, some pieces of chorioallantoic membrane were stained for acid phosphatase activity following the procedure advocated by ERICSSON and TRUMP (11) and employing cytidine monophosphate as the substrate.

Results

Entry into Cells of CAM

After incubation at 4° C for one hour, a few virus particles could be found closely associated with the plasma membrane but no intracellular particles were ever located. Particles were also seen attached to the plasma membrane on warming the preparations to 37° C.



Figs. 1 and 2. Active virus and CAM incubated for 1 hour at 4° C followed by incubation at 37° C either for 40 minutes (Fig. 1) or 20 minutes (Fig. 2). The virus in 1 a appears to be penetrating the cell membrane but after tilting the section through 36°, the particle is seen to be on the membrane surface (Fig. 1 b). Virus envelope in Fig. 2 a appears to be fusing with the cell membrane but after tilting the section through 30° (Fig. 2 b) the viral and cell membranes appear separate. The bar represents 100 nm

Occasional particles gave the appearance of melting their way through the plasma membrane. On analysis with the goniometer stage of the electron microscope, these appearances could be demonstrated to be due to the tangential plane of the section (Figs. 1 and 2). No positive evidence of the viral membrane fusing with the plasma membrane was found, such as the demonstration of continuity of virus and cell membrane.

After warming the chorioallantoic membrane preparations, virus particles were found in the process of being engulfed by viropexis (Fig. 3). On warming for 5 minutes, virus particles with intact envelopes were occasionally observed inside vacuoles whilst after 10 minutes the frequency of such observations increased considerably (Fig. 4). In CAM preparations that had been incubated for 30 minutes with a suspension of virus and ferritin (Fig. 5), ferritin particles were detected in vacuoles together with virus. Control samples incubated for up to one hour with ferritin alone showed no sign of ferritin uptake.

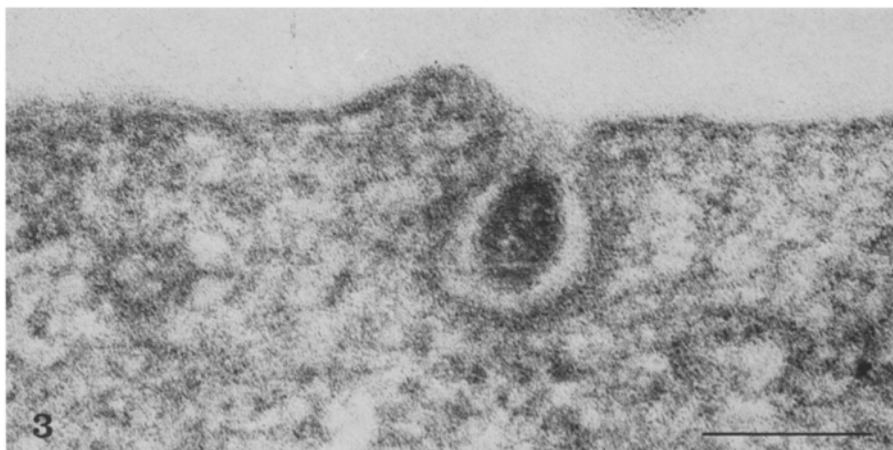


Fig. 3. Active virus and CAM incubated for 1 hour at 4° C followed by incubation at 37° C for 30 minutes. A virus particle in the process of being engulfed by viropexis may be observed. The bar represents 100 nm

After 20 minutes at 37° C, particles were frequently observed which appeared to be in the process of being pocketed by the vacuolar membrane (Fig. 6). Alternatively this may be interpreted as the vacuole coalescing with the virus. Cytoplasmic electron dense particles were occasionally observed in the vicinity of vacuoles (Fig. 5), but it was difficult to identify these unequivocally as virus particles. Hence it was not possible to resolve the later events in virus uncoating. Particles were still found attached to the cell membrane and inside vacuoles after warming for 60 minutes. Histochemical tests for the presence of acid phosphatase showed no staining of those vacuoles containing viral particles while lysosomes were intensely stained in other regions of the cell.

Internalization of virus neutralized by antibody prior to incubation with the CAM was not observed. Virus uptake was not seen in those preparations incubated with active virus for 1 hour at 4° C before being treated with antiserum and

subsequently warmed. In an attempt to improve the sensitivity of the system, the warm incubations were continued for 17 hours after the cold incubations in order to allow the replicative cycle to proceed. Thus one initial infectious particle should result in the generation of a large number of new virus particles which could then be detected by electron microscopy. After this prolonged incubation, numerous virus-containing vacuoles were located in those CAMs which were not treated with antiserum. Similar virus-containing vacuoles were not found in CAMs subject to incubation with virus for 1 hour at 4° C followed by treatment with antiserum for 1 hour at 4° C and finally incubated for 17 hours at 37° C. Likewise, no virus was found in those samples incubated with virus that had been inactivated by antiserum prior to being applied to the CAM.

Heat inactivated virus could not be located either attached to the cell membrane or inside vacuoles after 30 minutes incubation at 37° C.

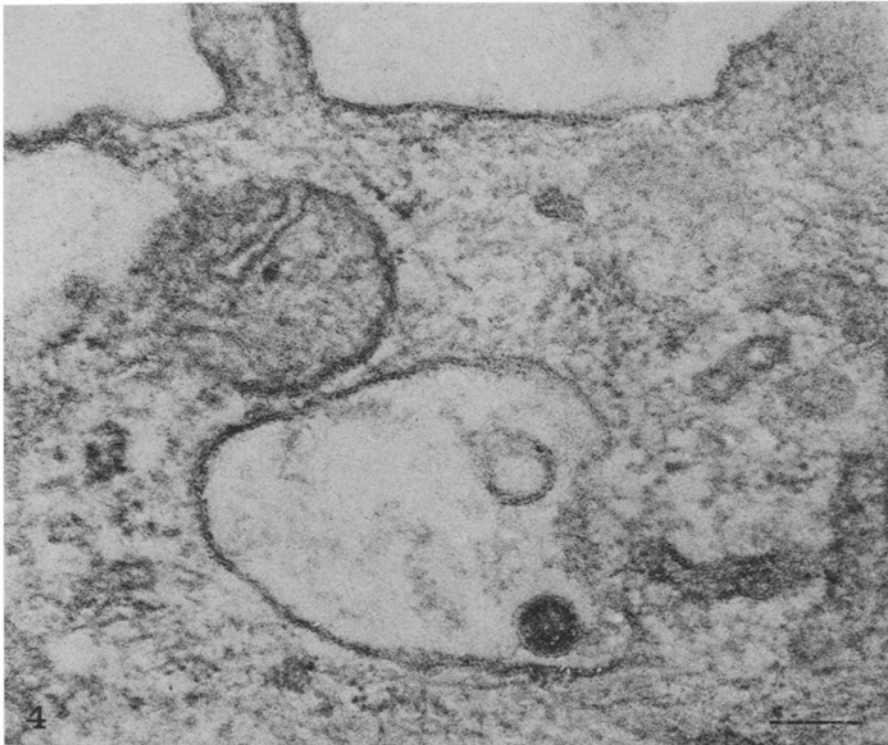


Fig. 4. Active virus and CAM incubated for 1 hour at 4° C followed by 10 minutes at 37° C. Virus particles with intact envelopes can be seen inside vacuoles. The bar represents 100 nm

Entry into Chicken Kidney Cells

Plaque formation on primary chicken kidney cells were carried out in order to give an estimate of the number of infectious virus units attaching to and entering the cells. Virus was adsorbed onto monolayers of CKC for 90 minutes

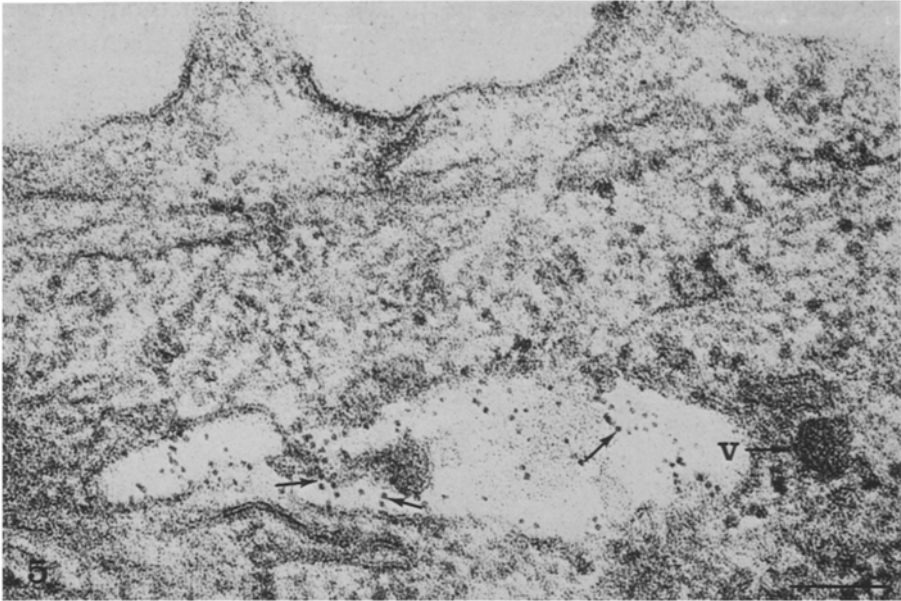


Fig. 5. Active virus and ferritin incubated with CAM for 1 hour at 4° C followed by 30 minutes at 37° C. Ferritin particles (arrows) and virus are found together inside vacuoles. An electron dense particle (V) which may be a virus particle, can be observed in the cytoplasm adjacent to the vacuole. The bar represents 100 nm

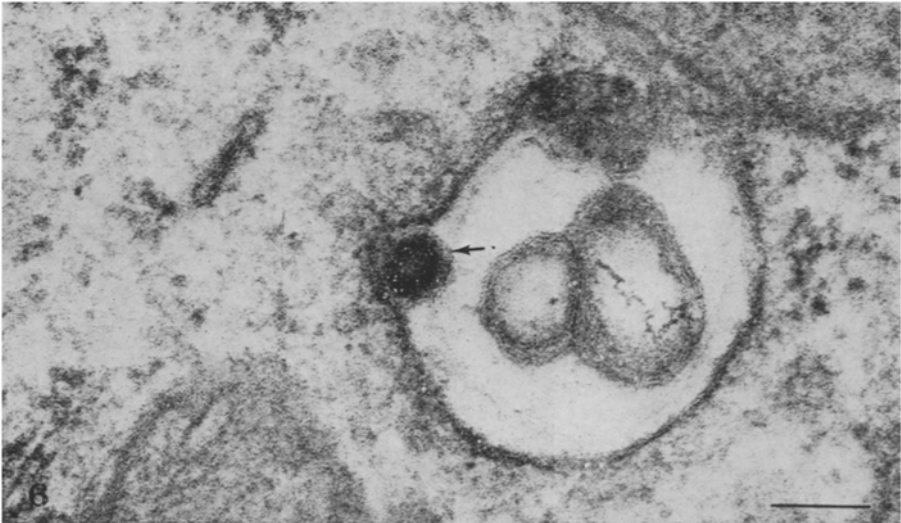


Fig. 6. Active virus incubated with CAM for 1 hour at 4° C followed by incubation at 37° C for 30 minutes. Virus particle (arrow) attached to the vacuolar membrane appears to be in the process of being pocketed by the membrane

at either 4° or 37° C, as described previously in Materials and Methods, and then subjected to one of three different procedures. Direct overlay without removal of the inoculation fluid gave the maximum number of plaques obtainable and this was compared with the plaque number obtained when the cell sheet was washed before being overlaid. It may be seen from Table 2 that only about one third of the available virus remained attached after washing the cell sheet. However, attachment was as efficient at 4° as at 37° C. Other dishes had antiserum added to neutralise extracellular virus before the overlay was added. There was no significant difference between the plaque numbers obtained for virus adsorbed at 37° C and either washed or neutralised, suggesting that at 37° C virus uptake must have been rapid once attachment had occurred. However, the cell layers to which virus had been adsorbed at 4° C and then neutralised showed a significant drop ($P < 0.001$) in the plaque number compared with both its 37° C equivalent and the 4° C adsorbed and washed cells. Thus although virus attachment is as efficient at 4° as at 37° C, the process of uptake into the cells has been much reduced at 4° C. To a fourth set of cells was added virus which had been previously mixed with the antiserum; this gave a control count of plaques to test the efficiency of neutralisation by the antibody. The plaques were almost totally abolished, and this number was significantly less ($P < 0.001$) than that in the experiment where virus was adsorbed at 4° C and then neutralised. Therefore there was a genuine uptake of virus into cells at 4° C, although at a much lower rate than at 37° C.

On examination of CKC by electron microscopy, attached virus was sparsely distributed along the cell membrane both in those preparations warmed to 37° C and in preparations only subject to a cold incubation. Internalized virus was not found in cells that had only been incubated for 1 hour at 4° C. This was exactly parallel to the situation seen in the CAM preparations.

Discussion

According to DALES (8) attachment is temperature independent whilst penetration is temperature dependent in most animal virus host cell systems. By electron microscopy adsorption of IBV onto CAM and CKC was seen to occur both at 4° and 37° C but virus entry was not observed until preparations were warmed. By the plaque counting system, we found that virus adsorption was similar at both temperatures; we also detected entry at 4° C, although this was considerably enhanced at 37° C. This apparent discrepancy is presumably a reflection of the sensitivity of the two different techniques employed.

Studies employing ferritin suggested that attachment of virus to the cell membrane stimulates viropexis, thereby initiating a cell mechanism for uptake of surface-attached particles. Similar findings to these have been reported for influenza virus entry into CAM (10).

Heat inactivation of IBV prevented its attachment and uptake by viropexis. This situation contrasts with heat inactivated influenza virus which become attached to the cell membrane and subsequently taken up by viropexis (10). Inactivation at 56° C for 30 minutes also abolished haemagglutination by IBV-Massachusetts and IBV-Connecticut (R. W. BINGHAM, M. H. MADGE, and D. A. J. TYRELL, unpublished results) and it is possible that a thermally unstable protein

is involved in the attachment to both CAM cells and erythrocytes. These findings support the view that attachment initiates viropexis.

Virus may enter susceptible cells by fusion of viral and cell membranes (1, 9) by viropexis (5, 6, 10), or in certain cases by both these routes (12, 13). Our observations demonstrated that viropexis was the main mode of uptake of IBV into cells of the CAM. Evidence to support this conclusion was derived from observations of particles in the process of being engulfed, from the presence of viruses with intact envelopes inside cytoplasmic vacuoles and from the absence of any evidence for the fusion of viral and plasma membranes.

In earlier experiments by STINSKI and CUNNINGHAM (18), employing radio-labelled IBV and embryonic chicken kidney cells, virus was adsorbed at 4° C, treated with antiserum, warmed to 37° C and subsequently found to become internalized. No evidence to support the uptake of antibody treated virus into CAM was found in the present experiments.

With the exception of the reoviruses (7, 16), successful entry leading to replication is not dependent on lysosomal enzymes in most viruses (8). In the present studies, acid phosphatase, a lysosomal enzyme, was not found closely associated with vacuoles containing viral particles; however, it is possible that some lysosomes stained with the acid phosphatase reagent may have contained virus that was obscured by the stain. It is likely that some particles failing to pass along the normal route of infection end up in lysosomes and are there inactivated and degraded.

The study of DOURMASHKIN and TYRRELL (9) described influenza virus penetrating between the bimolecular leaflets of the vacuolar membrane in order to gain access to the cell cytoplasm. Although in the present work many sections were observed in which the vacuolar membrane appeared to be actively pocketing viral particles, it was not possible to elucidate the precise mechanism by which the virus gained access to the cell cytoplasm.

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