

Effect of pH on the Growth and Cytopathogenicity of Avian Infectious Bronchitis Virus in Chick Kidney Cells

By

D. J. ALEXANDER and M. S. COLLINS

Poultry Department, Central Veterinary Laboratory, New Haw, Weybridge,
Surrey, England

With 7 Figures

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Summary

The growth of avian infectious bronchitis virus (IBV) in chick kidney cells at different pH values in the range 6.0—9.0 demonstrated that although the virus was released at a much faster rate at the higher pH values the titre tended to drop more quickly. At the acid pH values the virus was released more slowly but reached a maximum titre similar to that at the higher pH values and showed only minimum reduction in infectivity up to 49 hours post inoculation.

The stability of virus in tissue culture medium was shown to be directly related to pH between pH 6.0—8.0, being more stable at the acid pH values.

The degree of cytopathogenicity induced in chick kidney cells following infection with IBV was directly related to the pH at which the cells were incubated, occurring earlier and more extensively in cells at the higher pH values.

Cell macromolecule synthesis in chick kidney cells was inhibited following infection with IBV and was apparently due to cell damage and death.

Introduction

The coronaviruses in general, and avian infectious bronchitis virus (IBV) in particular, show a remarkable fastidiousness in their ability to grow in tissue culture systems. IBV requires initial isolation in chick embryos followed by a gradual adaptation to tissue culture which often involves several passages. Even after this procedure IBV strains have only been shown to grow in primary avian cells (8), primary monkey kidney cells (13, 20) and in VERO cells if first adapted to mouse brain (10).

Chick embryo kidney (CEK) cells have been the most popularly used cells for IBV studies (8) mainly due to the marked cytopathic effect (CPE) produced in these cells. AKERS and CUNNINGHAM (1) describe the CPE in CEK cells as the formation of syncytia which eventually become necrotic and detach from the glass,

the development of syncytia being sequentially and directly related to the release of infective virus. Plaques are formed in CEK cells following the necrosis of syncytia (8).

CHURCHILL (7) showed that CPE, involving syncytial formation, was induced by IBV in adult chick kidney (CK) cell cultures and suggested that since CK cells were more easily prepared they were more suitable for use in IBV studies. BRACEWELL (6) has since used CK cells for IBV plaque reduction assays.

POCOCK and GARWES (personal communication) have shown that the growth and stability of transmissible gastroenteritis virus (TGEV), a coronavirus of pigs, was greatly influenced by pH. Obviously such considerations are important in any study involving the production and storing of large quantities of virus. In the present paper we have examined the effect of pH on the growth, cytopathogenicity and stability of IBV grown in CK cells.

Materials and Methods

Virus

The Beaudette strain of IBV was obtained from C. D. Bracewell, Central Veterinary Laboratory, Weybridge, UK and had been passaged eight times in CK cells. A pool was grown in CK cells and this virus was used for infecting cells in all experiments.

Cell Cultures

Primary chick kidney (CK) cells were prepared from 4-week-old chicks as described (6). Except where stated pH was adjusted by the use of 15 mM HEPES buffer. Hanks' MEM maintenance medium, buffered with 15 mM HEPES when necessary, was used for radioisotope uptake experiments.

Estimations of Virus Growth

Confluent monolayers of CK cells in 75 cm² plastic flasks were inoculated with approximately 5 plaque forming units (PFU) of IBV per cell and incubated at 37° C. After one hour the monolayers were washed and covered with 20 ml maintenance medium. At the specified times after inoculation 0.5 ml samples were taken and infectivity estimated as plaque forming units (PFU) as described (6). Except where stated cells were inoculated at pH 7.0—7.2 (i.e. in media buffered with bicarbonate only) and media at the different pHs was added one hour after inoculation. Results are presented as the average of two experiments in which duplicate samples were taken.

Estimation of Cell Fusion

Cell coverslip cultures were infected with 10—20 PFU cell. At the specified times cell cultures were washed in phosphate buffered saline (PBS), fixed in methanol and stained with 10 per cent May Grünwald-Giemsa (19).

The extent of fusion was expressed as 'fusion events per cell' (15).

Radioisotopes

L-(4.5-³H)-leucine (56 Ci/mM) and (5-³H)-uridine (26.5 Ci/mM) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Incorporation of Radioisotopes

Incorporation of radioisotope labelled protein and ribonucleic acid precursors into trichloroacetic acid (TCA)—soluble and TCA—insoluble fractions of CK cells following a one hour pulse period was estimated as described (2).

Results

Virus Growth

When CK cells were inoculated with approximately 5 PFU IBV per cell in medium buffered with bicarbonate alone (i.e. pH 7.0—7.2) an increase in released virus was first recorded after three hours. The titres of virus in the medium increased logarithmically until 12—14 hours after inoculation reaching the maximum 14—16 hours after inoculation (Fig. 1).

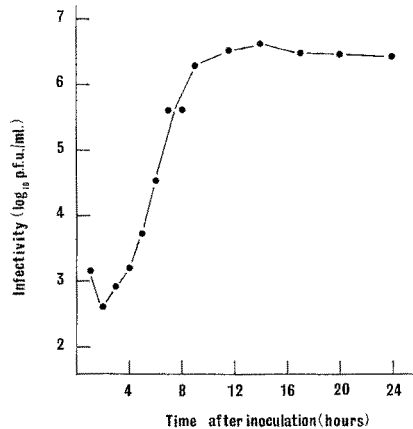


Fig. 1. Release of infective virus from CK cells inoculated with approximately 5 PFU IBV per cell. Medium was buffered with bicarbonate alone

Variations in the pH of media between pH 6.0 and 9.0 maintained by HEPES buffer produced marked effects on the growth of IBV (Fig. 2a—b), pH values outside this range tended to cause early death even in control cells. The onset of virus growth and rate of release of infective virus was directly related to pH between pH 6.0—8.0, at pH 9.0 titres tended to be slightly lower than at pH 8.0. At pH 8.0 the maximum titre of released virus ($10^{6.8}$ PFU/ml) was obtained between 10—15 hours after inoculation but fell off rapidly after this time. At acid pH similar maximum titres were only achieved 20—30 hours after inoculation. More noticeable was the maintenance of released virus titres at pH 6.0 or 6.5. By 49 hours after inoculation virus grown at pH 8.0 had fallen in titre from $10^{6.8}$ to $10^{1.8}$ PFU/ml whereas the drop in titre at pH 6.0 was from the maximum, $10^{6.68}$ PFU/ml (at 30 hours after inoculation) to $10^{6.22}$ at 49 hours post inoculation. The effect of pH on the yield of virus is best demonstrated by plotting yield against pH; this is shown in Figure 3 for virus infectivity at 11½ and 39 hours after inoculation.

In the experiments described above CK cells were inoculated and held at pH 7.2 for one hour prior to transfer to the stated pH. However initial adsorption of virus did not appear to be affected by differences in pH since the yield 20 hours after inoculation was independent of the inoculation pH. Cells maintained at the same pH values following the one hour adsorption period produced relatively constant yields at 20 hours post inoculation regardless of the pH of the inoculation media (Table 1).

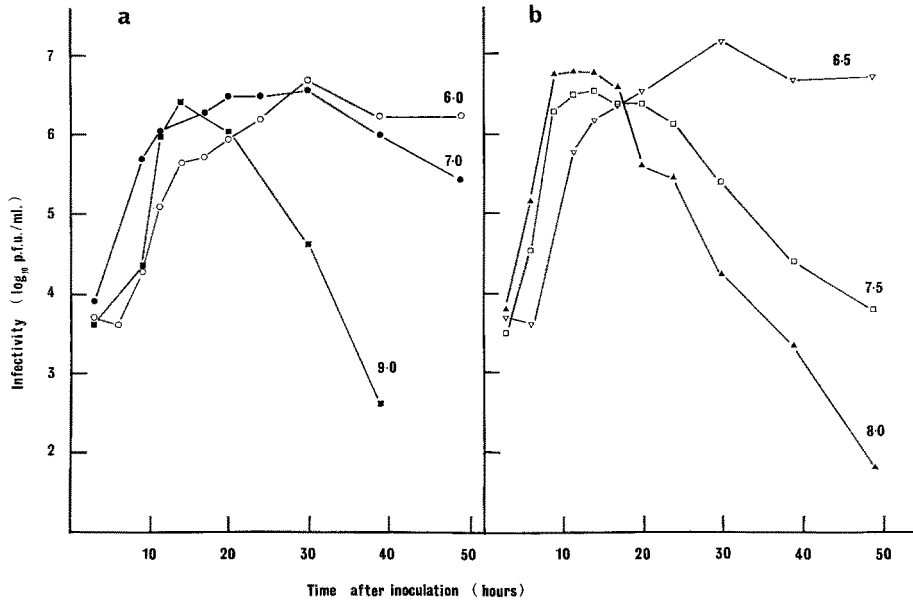


Fig. 2a, b. Release of infective virus from CK cells inoculated with approximately 5 PFU IBV per cell and maintained at the indicated pH from one hour after inoculation. Media were buffered to the respective pH values with 15 mM HEPES

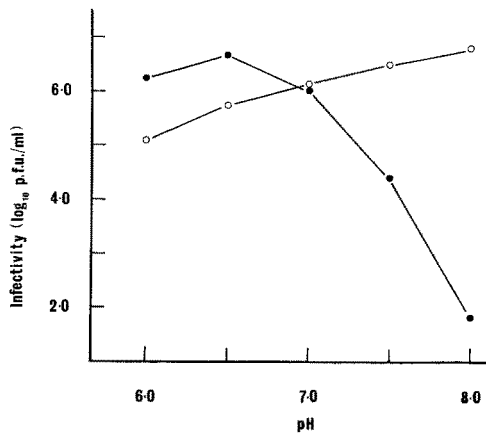


Fig. 3. Effect of pH on yield of IBV released from CK cells at 11½ hours (○) and 39 hours (●) after inoculation

The effect of pH on the stability of virus was estimated by measuring the infective titre before and after incubation for 24 hours at 4°, 23° and 37° C over the range pH 6.0—8.0 (Table 2). The virus was relatively stable at all pH values at 4° C. At 23° C the virus showed least loss in infectivity at the acid pH values, at pH 8.0 there was a decrease from $10^{6.0}$ to $10^{5.2}$ PFU/ml after 24 hours. The lack of stability of the virus was most marked at 37° C and was directly related to pH—there was a decrease in infectivity at pH 6.0 from $10^{6.16}$ to $10^{4.63}$ compared

to a drop in titre from $10^{6.0}$ PFU/ml to less than 5 PFU/ml at pH 8.0. Identical results were obtained if the virus was grown at pH 6.5 and adjusted to the different pH values or if the virus was grown at the respective pH.

Table 1. *Effect of pH at inoculation and incubation on virus yield*

Incubation pH	Inoculation pH	Yield at 20 hours post inoculation
6.0	7.0—7.2 ^a	6.65 ^b
6.0	6.0	6.63
7.0	7.0—7.2	6.57
7.0	7.0	6.75
8.0	7.0—7.2	6.43
8.0	8.0	6.60
9.0	7.0—7.2	6.04
9.0	9.0	6.32
7.0—7.2	6.0	6.80
7.0—7.2	7.0	6.54
7.0—7.2	8.0	6.95
7.0—7.2	9.0	6.79
7.0—7.2	7.0—7.2	6.85

CK cells were infected with approximately 10 PFU IBV per cell and held at the inoculation pH for one hour at 37° C. The cells were then washed twice with the inoculation media and incubated for a further 19 hours at the incubation pH

^a pH 7.0—7.2 represents media without HEPES buffer

^b Log₁₀ PFU/ml

Table 2. *Effect of pH on the stability of IBV*

Virus titre (log ₁₀ PFU/ml)	pH			
	Untreated	4° C ^a	23° C ^a	37° C ^a
6.0	6.16	6.19	6.08	4.63
6.5	6.30	6.30	6.00	4.48
7.0	6.10	6.07	5.89	2.40
7.5	6.09	5.93	5.67	0.70
8.0	6.00	6.00	5.15	<0.40

^a Treatment consisted of incubation for 24 hours at the specified temperatures

Cytopathic Effects

General

The first noticeable CPE in CK cells infected with IBV was the formation of small discrete syncytia, containing 3—4 nuclei, which occurred at 6—8 hours after inoculation at normal pH. The degree of CPE increased in parallel with virus release, the syncytia increasing in size to contain 20—40 nuclei (although some larger polykaryocytes were often present), by which time the nuclei had often become pycnotic and the syncytia greatly vacuolated.

At this stage syncytia tended to round-up into deeply stained cytoplasmic masses and detach from the glass. Because of the dark staining and fragmentation

of the nuclei it was usually impossible to assess the number of nuclei in polykaryocytes which had rounded-up. However massive syncytia containing hundreds of nuclei as seen in Newcastle disease virus infections of chick embryo cells (19) were never produced.

Although vacuolation of cells seemed to increase following infection, in agreement with the work of BERRY (5) this could not be regarded as typical CPE as preparations of uninfected CK cells frequently showed a high level of vacuolation.

The onset of CPE and the time taken to destroy the monolayer were directly related to the pH of the media (between 6.0—9.0), occurring earliest at the highest pH. This was so marked that by 18 hours after inoculation only occasional small syncytia could be seen at pH 6.0, while in cultures at pH 8.0 much of the monolayer had been destroyed and all cells were involved in large syncytia.

Cell Fusion

Quantitative estimations of the degree of cell fusion induced following infection of CK cells by IBV were difficult for two reasons: First, uninfected CK cell cultures possessed up to 20 per cent of binucleate cells, and secondly, estimations made later than 16 hours after inoculation were unreliable because of the deeply staining rounded syncytia and the fragmentation of their nuclei. Both these points tended to give a falsely low estimation of fusion but the technique could still be employed for comparative estimations of CPE. Typical results obtained by estimating fusion as the number of fusion events per cell are shown in Figure 4. It can be seen that increases in cell fusion are first detected towards the end of the log phase of the growth cycle of released virus.

To assess the effect of pH on cell fusion polykaryocytosis was estimated 16 hours after inoculation, following incubation in media buffered between pH 5.0—9.0.

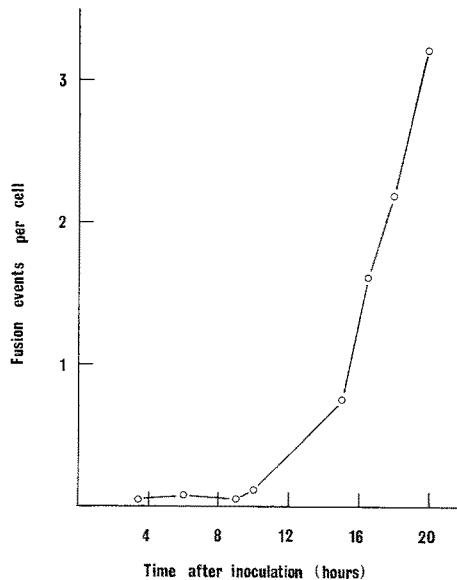


Fig. 4. Estimation of cell fusion in CK cells inoculated with 10—20 PFU IBV per cell, medium was buffered with bicarbonate alone

The degree of cell fusion was directly related to pH, no fusion occurring at pH 5.0 with a linear increase between pH 6.0—8.0, incubation at pH 9.0 producing the same level of fusion as at pH 8.0 (Fig. 5).

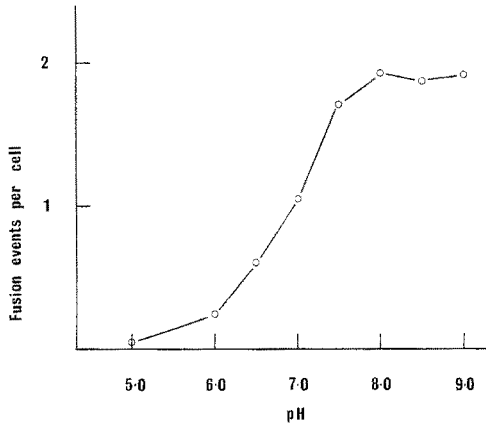


Fig. 5. Effect of pH on cell fusion in CK cell cultures inoculated with 10—20 PFU IBV per cell, media was buffered with 15 mM HEPES

Inhibition of Host Macromolecule Synthesis

Incorporation of (^3H)-uridine into a TCA-insoluble fraction of CK cells infected with IBV showed an initial increase compared to uninfected control cells (Fig. 6a). However from 9 hours after inoculation inhibition proceeded linearly with time so that by 24 hours after inoculation incorporation was only 10 per cent of the control.

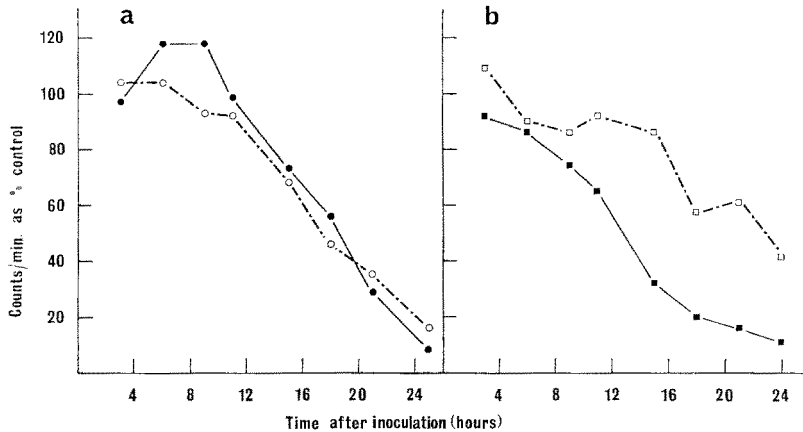


Fig. 6. Incorporation of (^3H)-uridine (a) and (^3H)-leucine (b) into TCA-insoluble (solid line, closed symbols) and TCA-soluble (broken line, open symbols) fractions of CK cells inoculated with 10—20 PFU IBV per cell. Coverslip cell cultures were treated with 1 ml of the respective radioactive precursors (1 $\mu\text{Ci}/\text{ml}$) one hour prior to the times specified. The results are expressed as per cent uninfected control cells. The medium was buffered with bicarbonate alone. Absolute incorporation: 100 per cent TCA-soluble; (^3H)-uridine: approx. 52,000 cpm/ml, (^3H)-leucine: approx. 4,000 cpm/ml. 100 per cent TCA-insoluble; (^3H)-uridine: 4,000 cpm, (^3H)-leucine: 1,000 cpm

TCA-soluble counts were unaffected until 12 hours after inoculation when inhibition of uptake began and proceeded at the same rate as inhibition of TCA-insoluble incorporation.

Uptake of (^3H)-leucine into both control and infected CK cells occurred only at relatively low levels. However, the results obtained (Fig. 6b) indicate that, like (^3H)-uridine incorporation, TCA-soluble and TCA-insoluble uptake of (^3H)-leucine was inhibited in parallel with virus release following infection.

These results suggest that inhibition of protein and RNA synthesis occurs in infected cells as a result of the onset of CPE and cell death and there is no specific mechanism by which IBV inhibits host cell synthesis. In this context it can be seen that increase in cell fusion and inhibition of (^3H)-uridine incorporation occur at identical rates. Further evidence that host macromolecule synthesis inhibition was related directly to CPE was the relationship between inhibition of (^3H)-uridine and (^3H)-leucine incorporation at 20 hours post infection and the pH of the media between pH values 6.0—9.0 (Fig. 7).

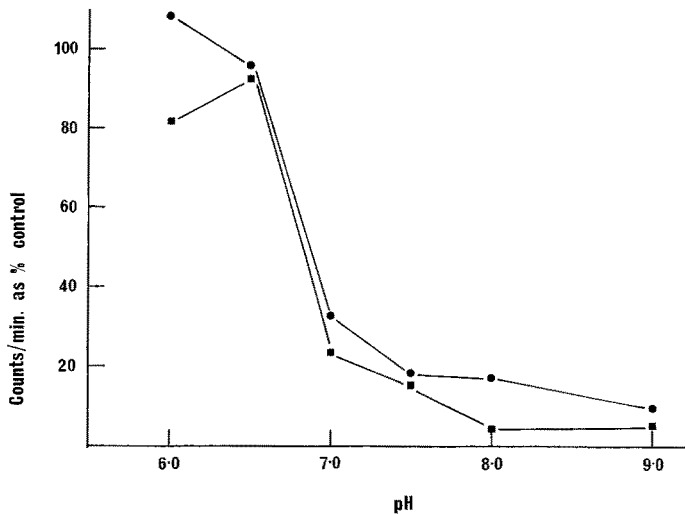


Fig. 7. Incorporation of (^3H)-uridine (●) and (^3H)-leucine (■) into TCA-insoluble fractions of CK cells inoculated with IBV and maintained at different pH values. Cells were labelled for one hour beginning 19 hours after inoculation as described for Figure 5, media were buffered with 15 mM HEPES

Discussion

Our studies on the stability of IBV in tissue culture media produced similar results to those obtained by POCOCK and GARWES with TGEV (personal communication). However other studies on IBV stability have produced conflicting results. Virus in cell culture fluid has been reported to be more labile at pH 2.9—3.1 than pH 7.1—7.3 following incubation for 4 hours at room temperature (11) but more labile at pH 11.0 than pH 3.0 following 30 minutes incubation at 4° C (21). CUNNINGHAM and STUART (9) reported an optimum of pH 8.5 for survival of IBV in phosphate buffer at 4° C and JORDAN and NASSAR (16) who investigated the

stability of IBV in water showed values of pH 5—9 had little effect on IBV stability but that least fall in titre occurred at pH 9. These apparent discrepancies are difficult to explain but may relate to the different conditions used or differences in the IBV strains used.

The growth cycle of IBV, in CK cells at pH 7.0—7.2, recorded in this study was basically similar to the growth in chick embryo kidney cells (1, 17). Virus growth, measured by released infectivity, occurred at the fastest rates in cells at the higher pH values over the range pH 6.0—9.0 but the yield at any given pH was dependent on the time of harvest, presumably due to the lack of stability at high pH since the peak virus titre was approximately the same at all pH values. For incubation periods of 24 hours or more pH 6.5 was the optimum for virus yield.

Apart from the lack of 'massive' syncytia, IBV-induced CPE was very similar to that induced in chick embryo (CE) cells by NDV (2, 19). However GALLAHER and BRATT (14) have reported a marked pH optimum of pH 8.2 for fusion of CE cells from within by NDV, whereas CK cells infected with IBV showed a linear increase in the degree of fusion between pH 5.0 and 8.0, which levelled off from pH 8.0 to 9.0. It would seem likely from our results with IBV that increase in syncytial formation at high pH is directly related to the increase in virus growth. However GALLAHER and BRATT (14) report that an increase in syncytial formation at high pH is not necessarily related to an increase in the growth rate of NDV. Caution should be used in making direct comparisons of IBV with other cell fusing viruses since different mechanisms may be involved. For example, it has been suggested (11) that virus-induced cell fusion occurs following the modification of the cell surface membranes by incorporation of virus specified products, but morphogenesis studies with IBV (4) have failed to show any budding from the surface of infected cells and with other coronaviruses no viral antigens could be detected in the membrane with ferritin-labelled antibodies (18).

In conclusion, our results indicate that IBV grown in CK cells is like another coronavirus, TGEV, in its extreme sensitivity to pH changes (POCOCK and GARWES, personal communication).

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Authors' address: Dr. D. J. ALEXANDER, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, England.

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