

STUDIES ON BOVINE HERPESVIRUSES. PART 1. ISOLATION AND CHARACTERIZATION OF VIRUSES ISOLATED FROM THE GENITAL TRACT OF CATTLE

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ABSTRACT

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Herpesviruses, previously isolated from cattle (Theodoridis, 1978), were further studied and provisionally placed in the bovid herpesvirus 4 (BHV-4) group.

Major differences were found between IBR-IPV (BHV-1) and BHV-4 virus strains. In MDBK cells, all BHV-4 strains started growing at the edges of the culture, the process progressing slowly until destruction of the cells was complete by the 10th day. BHV-4 strains failed to induce neutralizing antibodies in cattle, goats and rabbits. Only the addition of mineral oil adjuvant induced neutralizing and complement fixing antibodies in goats. BHV-1 strains, in contrast, produced very potent antisera in all these systems. Cross-neutralization tests indicated the existence of 2 distinct serological groups representing BHV-1 and BHV-4. The BHV-4 strains appear to be interrelated and they could not be grouped. A BHV-1 strain showed fixation of complement with the antisera of 6 BHV-4 strains.

Electron micrographs showed an accumulation of nucleocapsids in the cytoplasm and an early release of virus particles due to cell destruction. Variation in incubation temperature had a significant effect on the particle formation. At lower temperatures, the number of enveloped particles in the cytoplasm increased.

On the basis of the characteristics uncovered in this study, it is possible that all the BHV-4 strains represent one and the same virus which has undergone certain biological changes, thus illustrating a phenomenon which appears to be a characteristic of the herpesviruses.

INTRODUCTION

Herpesviruses have been isolated from man, domesticated mammals, birds, reptiles and fish (Fenner, 1976). Pathological changes have resulted in some species. The diseases caused by herpesviruses can result in economic losses. Examples are Aujeszky's disease in pigs; equine rhinopneumonitis in horses; infectious laryngotracheitis in poultry; and infectious bovine rhinotracheitis in cattle, infectious pustular vulvovaginitis in cows and infectious balanoposthitis in bulls.

At the turn of the century, Storch (1910) and Parker (1910) reported on their studies on "exanthema coitale" of cattle and suggested that a bacterial infection was the cause. Zwick & Gminder (1913) proved that a bacteria-free suspension in saline, prepared from vaginal discharges, could produce vaginitis, but the agent failed to pass through a No. 5 Berkefeld filter. In the late 1920's, Reisinger & Reiman (1928) reported that the agent of coital exanthema was a filterable virus. They suspended the vaginal discharge obtained from natural and experimental cases in bouillon instead of saline and showed that the virus could pass through a porcelain Reichl and Chamberland L3 filter. A virus, isolated in cell culture by Kendrick, Gillespie & McEntee (1958), was associated with a vesicular exanthema of the vaginal mucous membranes of cattle accompanied by a purulent discharge. This syndrome was similar to that described by Reisinger & Reiman in 1928. The disease was subsequently named infectious pustular vulvovaginitis (IPV) by Kendrick *et al.* (1958).

In the 1950's an infectious disease of the upper respiratory tract of cattle appeared in feedlots and on dairy farms in the USA. The syndrome was named infectious bovine rhinotracheitis (IBR) and the virus was isolated and described by Madin, York & McKercher (1956).

Subsequently, it was demonstrated that the viruses of IPV and IBR were serologically identical (Gillespie, McEntee, Kendrick & Wagner 1959; McKercher, Straub, Wada & Saito, 1959).

The spectrum of diseases caused by the IBR-IPV virus widened when its involvement was demonstrated in conjunctivitis, encephalomyelitis and abortion in cattle

(Johnston, Simmons & McGavin; 1962; McKercher & Wade, 1964).

Viruses isolated from the respiratory and genital tracts of cattle causing either of the above described clinical symptoms were usually named IBR-IPV virus. Biological and biophysical studies did not reveal any fundamental differences between the IBR and IPV strains of viruses.

Electron microscopy revealed morphological characteristics (Tousimis, Howells, Griffin, Porter, Cheatham & Maurer, 1958; Liess, Knocke & Schimmelpfennig, 1960) similar to those of the B virus of monkeys (Reissig & Melnick, 1955). The morphology of IBR-IPV virus met the criteria of the herpesvirus group and it was therefore classified as the first bovine herpesvirus (Wildy, Russell & Horne, 1960; Armstrong, Pereira & Andrewes, 1961).

Recent studies using DNA restriction endonuclease "fingerprinting" of the DNA, however, allowed the differentiation between IBR-IPV virus strains (Engels, Steck & Wyler, 1981).

In Africa, a clinical syndrome involving the genital tract of cattle was reported by Daubney, Hudson & Anderson (1938). In bulls, epididymitis and orchitis and, in cows, vaginitis, cervicitis and endometritis were prominent symptoms, accompanied by dry peritonitis in both sexes. The affected animals became infertile or permanently sterile and had to be destroyed. This syndrome was later referred to as "epivag". A similar clinical syndrome reported in southern Africa in 1949, caused great economic losses to the cattle farming industry (Van Rensburg, 1949) and was listed as a notifiable disease in South Africa and Zimbabwe.

Maré & Van Rensburg (1961) examined cattle on farms in the Transvaal where infertility, sterility and abortions were causing problems, and during the course of that investigation a number of viruses were isolated. Further study revealed that IPV was present on many farms in the Transvaal. Cows experimentally infected with this virus developed fever, severe anterior but mild posterior vaginitis, cervicitis and a copious tenacious mucopurulent discharge which persisted for several weeks. The clinical picture was regarded as yet another manifestation of the IPV-IBR group of viruses. However, no disease of the respiratory tract could be produced (Maré, 1964).

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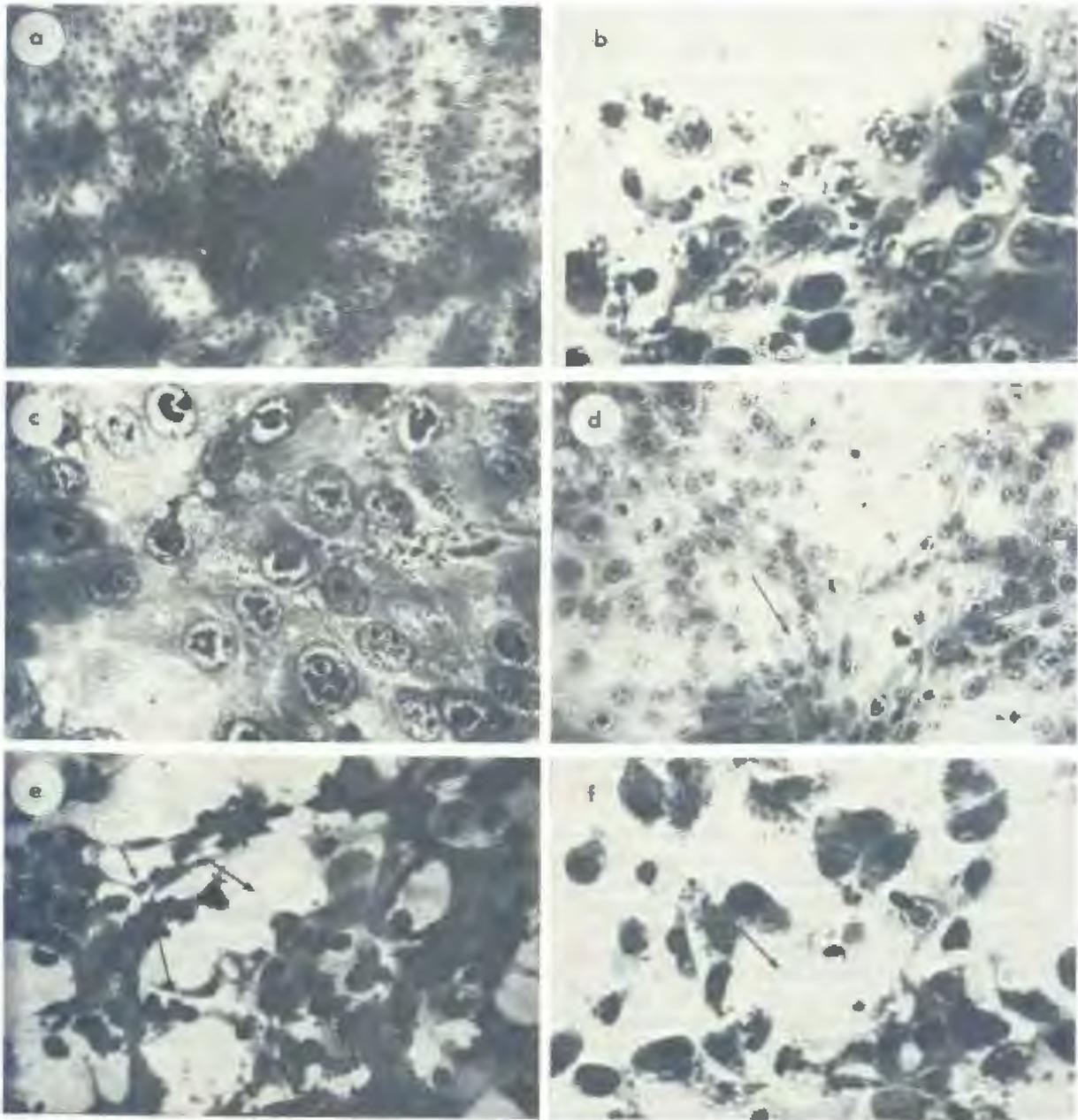


FIG. 1 a. Lesion in FCK cells caused by BHV-4 isolates
 b. Cells in the CPE areas are not pycnotic despite advanced CPE
 c. Intranuclear inclusions were present in cells of areas without visible CPE
 d. The cells around a focus show some tendency to orient towards it
 e. Cell destruction and open spaces caused by BHV-1 virus. Notice the pycnotic cells
 f. The centre of a focus at the early stage of infection with BHV-1 virus. Cells have detached at an early stage of CPE

Sterility and infertility are considered a serious problem in ranch cattle in South Africa. A large number of bulls are destroyed annually because they become sterile within 3-6 months after introduction into an infected herd. Many farmers consider the culling of these bulls a necessity for combating the spread of the disease.

The various viruses isolated from cases of sterility and infertility over the years have been shown to be herpesviruses and they fall into 2 main serological groups. One group is related to IBR-IPV and the other not (Maré & Van Rensburg, 1961; Theodoridis, 1978). The viruses isolated by these authors were divided into fast and slow growing viruses according to the rate of development of the cytopathic effect in tissue culture. Cows experimentally infected with the 2nd group of viruses also produced different clinical symptoms, consisting of both anterior

and posterior vaginitis, but without vesicle formation (Theodoridis, 1978). IBR-IPV virus has also been isolated from cases of epididymitis by Swanepoel & Christie (1972) and Kaminjolo, Nyaga, Omuse & Mutiga (1975).

The International Committee for the Taxonomy of Viruses has agreed that the herpes group should be given the taxonomic rank of family (Matthews, 1979). The definitive group description of the Herpesviridae accepted was as follows: Herpesviruses contain a double-stranded DNA with a molecular mass of 54 to 92×10^6 and a G + C content 57 to 74 %, the virus particle measuring about 100-150 nm and the nucleocapsid 100-110 nm diameter. The capsid is icosahedral with 162 hollow capsomeres of 10 nm diameter. It has a lipid-containing membrane and is therefore sensitive to lipid

TABLE 1 Origin and identification of virus isolates used in this study

Original designation	Lab. identification	Plaques utilized	Type of sample	Clinical signs in donor	Name of investigator
Epiv. 1/67	H1	2 & 3	Vaginal swab	Vaginitis	A. Theodoridis 1967
Epiv. 1/70	H2	2 & 4	Tissues of epididymis + testes	Epididymitis + orchitis	A. Theodoridis 1970
G 26	H3	4	Vaginal swab	Anterio-cervical vaginitis	J. Maré 1961
Epiv. 1/57	H4	4	Vaginal discharge	Vaginitis	K. E. Weiss 1957
C 375	H5	1	Vaginal swab	Vaginitis	J. Maré 1961
C 2864	H7	2	Vaginal discharge	Posterior vaginitis	H. Hellig 1965
Cotyl. (Herpes)	H8	1	Cotyledon tissue	Abortion at 6 months	A. Theodoridis 1970
P10	H9	1	Vaginal discharge	Anterior vaginitis	J. Maré 1961
B2	H10	2	Semen	Epididymitis	J. Maré 1961
FH 335	H11	4 & 6	Vaginal discharge	Vaginitis	J. Maré 1961
Bantam	H12	1	Semen	Epididymitis	J. Maré 1961
B 78	H13	1	Vaginal swab	Posterior vaginitis	J. Maré 1961
Epiv. 1/61	H14	1	Semen	Epididymitis	J. Maré 1961
Epiv. 2/61	H15	1	Semen	Epididymitis	J. Maré 1961

solvents. The DNA contributes about 7 % of the particle mass and the bouyant density (CsCl) is 1,27–1,29 g/cm³. Development begins in the nucleus and is completed by the addition of protein membranes as the virus passes into the cytoplasm. Intranuclear inclusion bodies are formed.

In this study, only some of the above-mentioned characteristics were investigated; in particular, those considered essential in a preliminary characterization of the isolates.

MATERIALS AND METHODS

Cell cultures

Primary foetal calf kidney (FCK) and testis (FCT) cells for the isolation of the viruses from field samples were prepared by cold trypsinization of the fragmented organ (Paul, 1973). Using the same technique, foetal lung cells (FLC) were prepared from 32 cm long calf foetuses. All these cell types were used for no more than 5 subcultures. The monolayers were prepared in tissue culture tubes and kept in rolling drums in an incubator at 37 °C.

A locally developed foetal calf kidney cell line at the 200th and 210th subculture was used for plaque purification of the viruses as well as for some of the early tests, including preparation of the initial stock viruses, haematoxylin-eosin staining and part of the neutralization tests. Baby hamster kidney cells (BHK-21 clone 13) (Macpherson & Stoker, 1962), African green monkey (VERO)* and a line of bovine kidney (MDBK)** cells were also used for a comparative susceptibility study.

Stock cell cultures were maintained in Roux flasks and transferred to tubes or Petri dishes as required. All cells were cultured in Eagle's modified medium (Macpherson & Stoker, 1962), containing in addition 10 % bovine serum, 200 IU of penicillin, 200 µg of streptomycin, 200 IU colistin sulphate and 2,5 µg of amphotericin B per ml.

Virus strains

Fifteen strains of virus were included in this study. Their origin is summarized in Table 1, where it can be seen that 3 of the isolates were known to be IPV-IBR viruses. The original primary isolation of all these viruses was undertaken in the 2nd–5th FCK subculture or the line of FCK cells.

* Yasumura, T. & Kawakita, Y. 1962. Chiba University of Chiba, Japan

** Maden Darby bovine kidney, supplied by the American Type Culture Collection

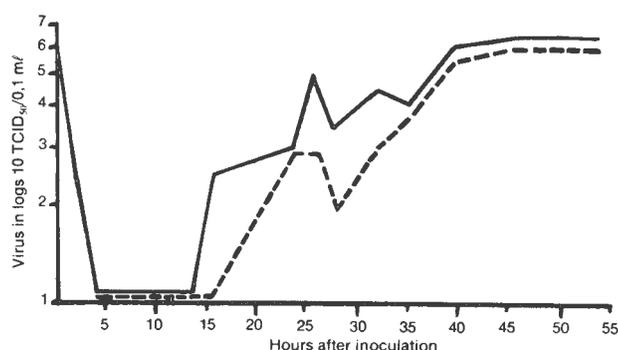


FIG. 2. Growth curve of H2/4 in FCL cells

— concentration in cells
- - - - - concentration in fluid media

The strains were identified by the letter H and a number from 1–15, since they had been previously identified as herpesviruses. In addition, the plaque number selected for the serological and pathogenicity studies was added.

Herpesvirus 1 was isolated from a vaginal swab taken from a cow which developed severe posterior vaginitis after being served by a bull. On this farm vaginitis was observed in many cows after being served by this bull.

Herpesvirus 2 was isolated in tissue culture from samples of epididymis and testis tissue. The bull from which this sample was collected had developed epididymitis and orchitis some months previously and was regarded as sterile. Several other bulls showed similar symptoms in this herd and the conception rate of the cows was very low.

Herpesvirus 3 and Herpesvirus 10 were collected from the same farm, where vaginitis had been present for several years and where the affected cows seldom conceived. Herpesvirus 3 was isolated from the vaginal exudate of a cow while Herpesvirus 10 was recovered from the semen of a recently introduced bull.

Herpesvirus 4 was recovered from the vaginal exudate of a virgin heifer. In this herd several heifers had shown vaginal discharge.

Herpesvirus 5, herpesvirus 12 and Herpesvirus 11 were isolated from cattle comprising various breeds on a large cattle farm. During the plaque purification of virus FH335 (H11) (Maré & Van Rensburg, 1961), 2 distinct viruses were obtained from plaque numbers 4 and 6 (Table 1). Subsequently, the same sample at an earlier subculture (FH335, FCK gen.7) was again subjected to plaque purification, but on this occasion it failed to produce evidence of a mixed population. There is a possibility that plaque number 6 was a laboratory contaminant.

Hellig (1965) isolated a number of viruses which were shown to be antigenically related to the virus IBR-IPV. When one of the isolates was subcultured in 1969, a virus antigenically different from IBR-IPV was identified and designated H7, but unfortunately there was not enough original material left to repeat the plaque purification of this strain. This virus originated from the vaginal exudate of a cow from a herd in which the majority of cows showed an acute pustular vulvovaginitis. The bulls in this herd showed pustular balanoposthitis which regressed and healed within 14 days.

Herpesvirus 8 was isolated in cultures of FCK cells from the cotyledon tissue of a foetus aborted in the 6th month of gestation. Sporadic abortions had occurred in this herd where tests for *Brucella abortus* and *Campylobacter fetus* tests were negative. Out of 12 sera examined from cows with a history of abortion, only one contained antibodies to this virus.

Herpesvirus 9 was obtained from a cattle farm where the cows that had been served developed persistent vaginitis and showed a low conception rate and all the newly introduced bulls became sterile within 6 months. The above virus was isolated from a vaginal discharge of one of these cows.

Herpesvirus 13 was isolated in FCK line cell cultures from a vaginal swab of a cow showing posterior vaginitis. The mucous membranes were red and swollen and covered with a very thin layer of stringy pus. Sporadic abortions and vaginal discharge were reported in this herd.

Herpesviruses 14 and 15 were isolated from the semen of bulls and which originated from 2 widely separated farms reportedly showed epididymitis.

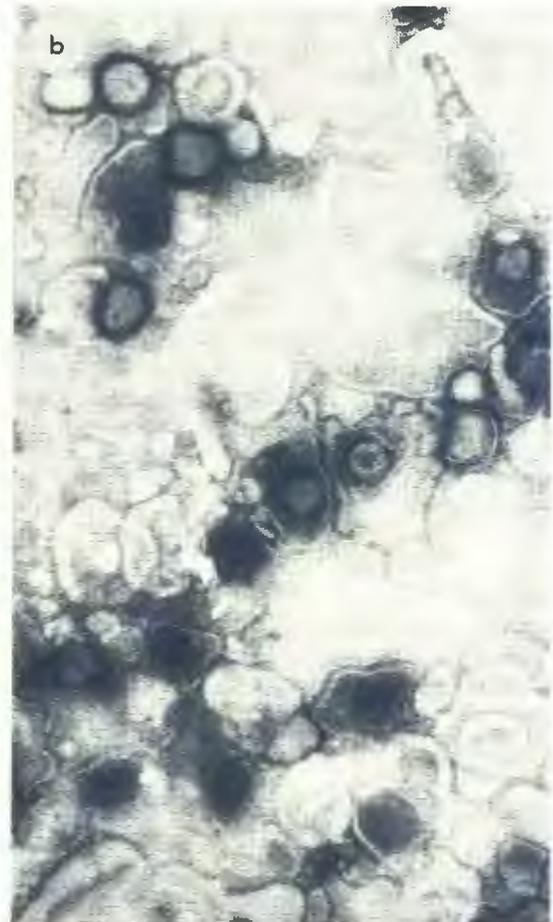
Virus isolation

Vaginal and preputial swabs were eluted in 2 ml of phosphate buffered saline (PBS) pH 7.2, containing 500 IU of penicillin and 500 µg of streptomycin per ml. The vaginal discharges, semen, uterus contents and heparinized blood were made up to a 10 % suspension in the antibiotic solution and held at 4 °C for at least 4 hours before being used to inoculate roller tube cultures.

Samples of tissue mass-measuring approximately 1 g were homogenized in 9 ml of antibiotic solution, clarified by centrifugation and kept at 4 °C. Confluent cell monolayers cultured in tubes were washed twice with Hank's BSS and inoculated with 0.2 ml of the tissue suspension. The inoculated tubes were rolled for 1 hour at 37 °C. Thereafter, the inoculum was decanted, the tubes washed twice with PBS and fresh maintenance medium again added. The tubes were examined daily under the microscope and the CPE was recorded.

Production of virus stock

Samples of viruses were diluted 1:100 in Eagle's medium and roller cultures of FCK, FCT, MDBK and foetal calf lung cells (FCL) not older than 48 hours were inoculated with 0.2 ml of inoculum. The virus was allowed to adsorb for 1 hour before the inoculum was decanted and replaced with 1 ml of maintenance medium. When CPE was advanced, the cultures were frozen and thawed twice and the contents of the tubes centrifuged at 1 500 × g for 10 minutes. The supernatant was lyophilized in 0.5 ml volumes in glass ampoules after a solution of bovine serum albumin had been added to give a final concentration of 0.5 %. The ampoules were sealed and stored at -20 °C.



Plaque selection

Monolayers of FCK line cells were cultured in 60 × 15 mm Petri dishes at 37 °C in an atmosphere containing 5 % CO₂. The confluent monolayers were washed twice with Hank's BSS, inoculated with an appropriate dilution of the virus and allowed to adsorb for 1 hour at 37 °C. The inoculum was aspirated and 4 ml of overlay added to each dish. The agar overlay was prepared as follows:

66 % Earle's salt solution plus sodium bicarbonate (NaHCO₃) solution to a final concentration 1,4 mg/ml, to which agarose was added at a final concentration of 0,5 %.

34 % Eagle's modified medium.

The cultures were observed daily for the development of plaques. The shape and size of the plaques were determined under an inverted microscope at a low magnification.

For purification of the strains, plaques were selected, removed by suction through a pipette and each plaque was suspended in 2 ml of Hank's BSS. Tubes with FCK line cell cultures were each inoculated with 0,2 ml of the plaque suspension.

Virus assay

Virus titrations were carried out in roller tube cultures of FCK cells. Tritrations were performed by standard procedures, using tenfold dilutions in Hank's BSS of the virus concerned. Four tubes per dilution were used, each receiving 0,1 ml of each respective dilution. The pro-

gress of cytopathic changes was recorded for 10 days, and end-points were calculated by the method of Reed & Muench (1938).

Serum virus neutralization test

Preliminary tests showed that the concentration of antibody in the various antisera was generally very low. As a result, a constant serum-virus dilution technique was selected and used throughout this study. For this test, serial tenfold dilutions of virus were prepared and held in an ice-water bath. Equal volumes of each dilution was then mixed with either undiluted or a 1:4 dilution of serum. The mixtures were then incubated for 1 hour in a waterbath at 37 °C. Four FCK roller tube cultures were inoculated with 0,2 ml of the serum-virus mixture, rotated for 1 hour at 37 °C, after which the inoculum was decanted and 1 ml of Eagle's medium added. The roller tubes were examined daily and the cytopathic effect (CPE) recorded. End-points were calculated for each serum virus mixture when 50 % CPE was observed in the tubes receiving the highest infective virus dilution in the control titration. The neutralizing index was calculated as the difference between the end-point of the virus titration and that of the virus in the presence of each serum.

Influence of complement on the neutralization reaction

The effect of complement on the virus-antiserum reaction was determined according to the technique described by Yoshino & Taniguchi (1965), who showed that antiserum-sensitized herpesviruses treated with complement tended to be neutralized more effectively and to a higher degree than the untreated virus. For this

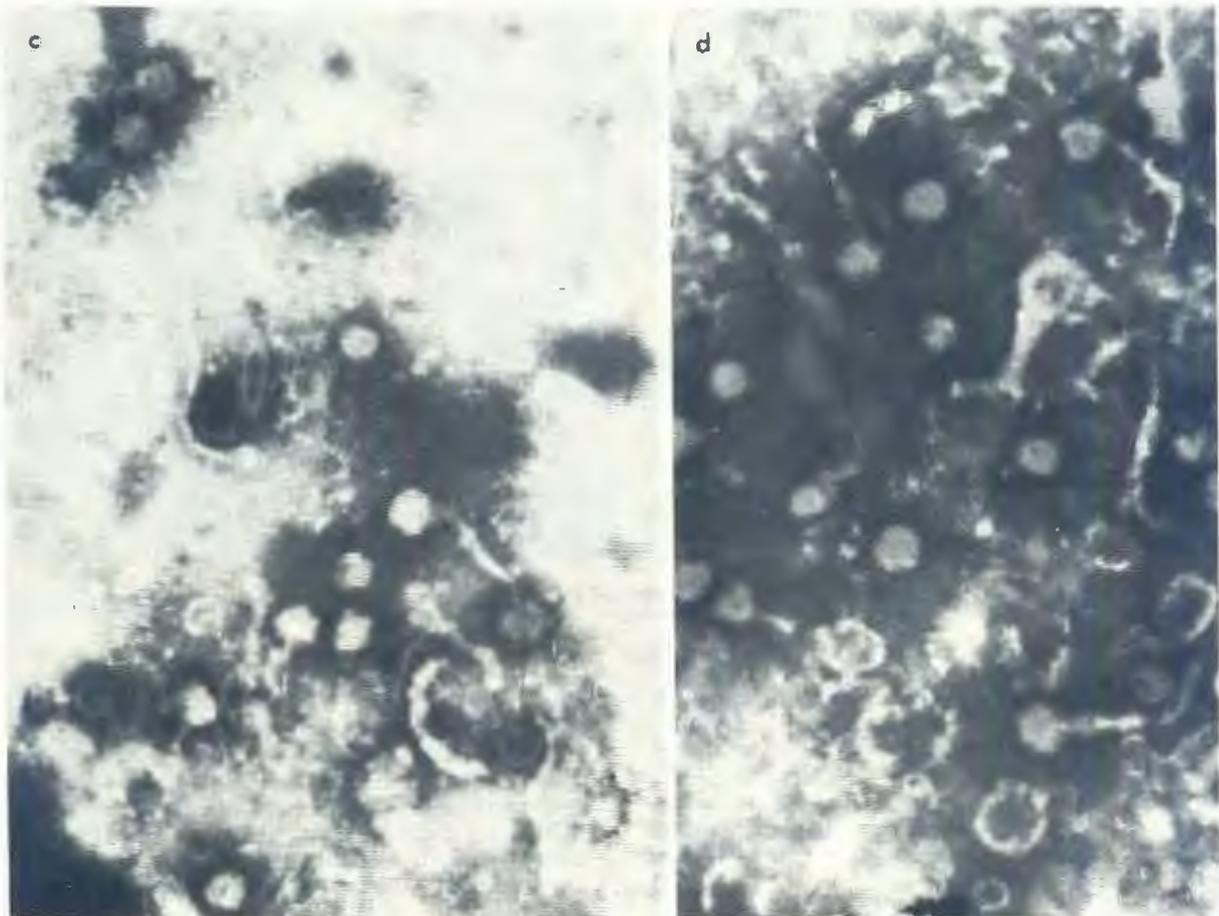


FIG. 3 a. Nucleocapsids of H2/4 from infected FCK cells incubated at 37 °C. Magn. 35×10^3
 b. Enveloped and naked nucleocapsids of H1 1/4 virus from infected FCK cells incubated at 37 °C. Magn. 22×10^3
 c. Enveloped and unenveloped particles of H2/4 incubated at 34 °C. Magn. 25×10^3
 d. Enveloped and unenveloped particles of H2/4 incubated at 32 °C. Magn. 25×10^3

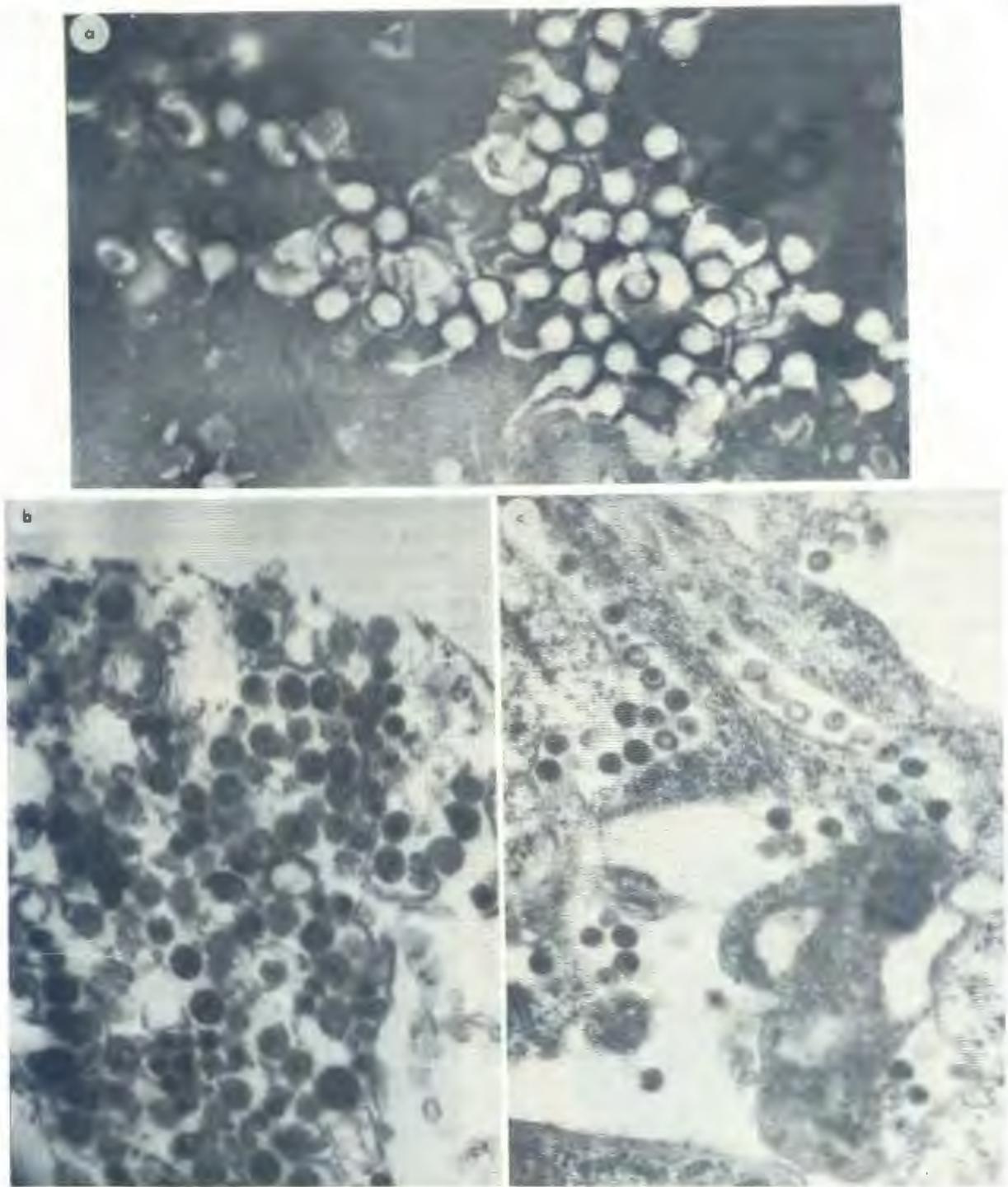


FIG. 4 a. H2/4 virus particles from FCL cells incubated at 37 °C. Magn. 25×10^3
b. H2/4 virus in sectioned FCL cells incubated at 32 °C showing an electron dense substance in the space between capsid and envelope. Magn. 30×10^3
c. H11/4 virus in sectioned MDBK cells incubated at 34 °C. Magn. 20×10^3

test, strains H2/4 and H11/4 with their homologous sera were selected. A duplicate series of serum-virus mixtures, including a control virus titration, was prepared as described above. After incubation for 1 hour at 37 °C., 0,5 ml of previously standardized complement was added to one of the series of mixtures. Roller tubes were then infected, as previously described, and the neutralizing indices finally determined.

Conduct of the complement fixation test

The complement fixation test (CF), undertaken with the specific sera prepared in goats and cattle, was conducted according to the method described by Cunning-

ham (1973). All the virus antigens included in the CF test were prepared in FCL cells in Roux flasks in an incubator at 34 °C. Uninfected cell suspensions were used as control antigen in the test. In the calculation of the results the highest serum dilution which fixed 50 % of the complement was taken as the end-point. The method of calculating the serum end-point was similar to that described by McIntosh, Haig & Alexander (1954).

Preparation of specific immune serum

Goats

Adult, nondescript goats were used. Two animals were injected with each virus. This virus was prepared

TABLE 2 Effect of protamine sulphate on plaque formation by BHV-4 viruses

Strain of virus	Titre		Mean plaque size (diam)		
	Untreated	Treated	Untreated	Treated	S.E.
			mm	mm	mm
H2/4	5,0*	6,5	0,6	0,6	0,5
H3/4	6,5	6,5	1,0	2,0	1,0
H5/1	6,5	5,0	1,0	1,5	0,5
H7/2	7,5	7,0	1,0	1,5	0,5
H8/1	6,5	5,5	0,5	1,0	0,5
H9/1	7,0	6,0	0,8	1,3	0,5
H10/2	4,5	5,0	0,6	1,0	0,4
H11/6	7,5	7,0	1,0	1,0	0,5
H12/1	6,5	7,0	0,4	1,0	0,6
H14/1	6,5	6,5	1,0	1,0	0,5
H15/1	6,5	4,5	0,6	1,2	0,6

* Log to base 10 TCID₅₀/ml
S.E. = Standard error

TABLE 3 Effect of IUDR on BHV-1 and BHV-4 viruses

Serogroup	Strain of virus	IUDR		Log inhibition
		Untreated	Treated	
BHV-1	H1/3	6,5*	4,5	2,0
BHV-1	H11/4	6,5	3,5	3,0
BHV-4	H2/4	7,5	1,0	6,5
BHV-4	H3/4	6,5	1,0	5,5
BHV-4	H4/4	5,5	0,5	5,5
BHV-4	H5/1	5,5	0,5	5,5
BHV-4	H7/2	5,5	0,5	5,5
BHV-4	H8/1	7,5	5,0	2,5
BHV-4	H9/1	6,5	1,0	5,5
BHV-4	H10/2	6,0	3,5	2,5
BHV-4	H11/6	6,0	3,5	2,5
BHV-4	H12/1	7,5	0,5	7,5
BHV-4	H14/1	6,5	1,0	5,5
BHV-4	H15/1	6,5	1,0	5,5
Control (RNA virus)	ECBO	6,5	6,5	0,5

* Log to base 10 TCID₅₀/ml

from roller tube cultures of FCK, FCL and MDBK cells infected with 0,2 ml of a 10⁻² dilution of each virus. When the CPE involved more than 50 % of the cells, they were harvested, frozen and thawed twice in a dry ice/alcohol bath and centrifuged at 1 500 × g for 10 minutes. The supernatant fluid was aspirated and stored at 4 °C. All these virus samples were titrated in roller tubes to confirm that the infective titre was not less than 10^{6.0} TCID₅₀/ml.

Equal volumes of each virus suspension and Freund's incomplete adjuvant were mixed together and each goat received 4 ml of the stable emulsion. To avoid tissue damage, 2 ml of inoculum was deposited in the muscles of each hind leg. Four weeks later, 2 ml of the virus suspension was injected intravenously and, 10 days after this 2nd injection, blood was collected. The H14/1 strain was used to prepare an antiserum to both pelleted virus from infected FCL cultures as well as whole suspension of similarly infected cultures. The antigen was injected intramuscularly with Freund's incomplete adjuvant into goats according to the method described above.

Rabbits

Adult albino rabbits from a closed breeding colony were used. A random sample of 5-7 rabbits were bled from the heart, and their sera were tested to determine their susceptibility to the viruses used in the study. Virus suspensions were prepared in a similar manner to that described for the immunization of the goats. Two rabbits were injected with 2 ml of each antigen, first intramuscularly and then by an intraperitoneal injection at the end of the 3rd week. Ten days thereafter blood was collected from the heart.

Cattle

Several of the isolates were also injected into cattle which had been shown to be free of neutralizing antibody. The viruses were cultured in FCK cell cultures and whole cultures were injected intramuscularly, intravenously and intravaginally. The animals were observed for clinical signs, and serum was collected 6 weeks after the last inoculation.

Reference antisera were prepared in goats and cattle against strain FH335 (Maré & Van Rensburg, 1961) and subsequently identified as H11/4, as well as against a strain of bovine ulcerative mammillitis. The former was taken to represent IBR-IPV (BHV-1) and the latter (BHV-2). These antisera were included in various tests as control sera or for the purpose of comparison.

From all these donors the blood which was collected was kept at 37 °C for 2 hours, then placed at 4 °C for several hours before being centrifuged at 2 000 × g for 30 minutes. The serum was aspirated aseptically, inactivated for 30 minutes at 56 °C and stored at -20 °C in 20 ml bottles. The pre- and post-inoculation sera were tested against the homologous and heterologous viruses.

Growth curve

FCL cultures in roller tubes were washed twice with PBS and each tube inoculated with 0,5 ml of a 1:100 dilution of virus strain H2/4 in Eagle's medium. Sufficient tubes were infected and incubated at 37 °C. The virus was allowed to adsorb for 2 hours. At periodic intervals 5 roller tubes were removed, the media pooled and the supernatant centrifuged at 2 000 × g for 10 minutes and stored at 4 °C. The cells remaining in the tubes were washed twice with PBS, 1 ml of Eagle's medium was added and thereafter frozen and thawed twice. When all the samples had been collected, they were titrated to determine virus infectivity. All the tubes were examined every 2 days under a light microscope. The CPE was recorded and correlated with the formation and release of infectious virus.

Effect of temperature of incubation

Two virus strains, H11/4 (rapid growing) and H2/4 (slow growing), were included in this test. FCL cells grown in Roux flasks were inoculated with 10 ml of virus suspension diluted 1:10 in Hank's BSS. The inoculated flasks were kept in an incubator at 37 °C for 1 hour and thereafter 90 ml of Eagle's maintenance medium was added. Flasks were incubated at 37 °C, 34 °C and 32 °C until the CPE exceeded 50 %. The medium was poured into centrifuge bottles and cell debris was separated by centrifugation at 1 500 × g for 15 minutes. The supernatant was removed and centrifuged at 25 000 × g for 1 hour. The pellets derived from this procedure were then resuspended in PBS (0,2 ml) and used to prepare grids for electron microscopy. The proportion of enveloped and unenveloped nucleocapsids was estimated by viewing 20 fields of each grid.

Effect of protamine sulphate

Protamine sulphate* at a concentration of 100 µg/ml of overlay was added to determine its effect on plaque size and the infective titre. The cultures were prepared from a line of FCK cells and the overlay was prepared in a manner similar to that previously described. Each virus was diluted so that a suitable dilution could be selected to measure the plaque size of individual, well-dispersed plaques. Ten plaques of both the controls and treated preparations were counted.

* Sigma Chemical Company, Missouri, USA

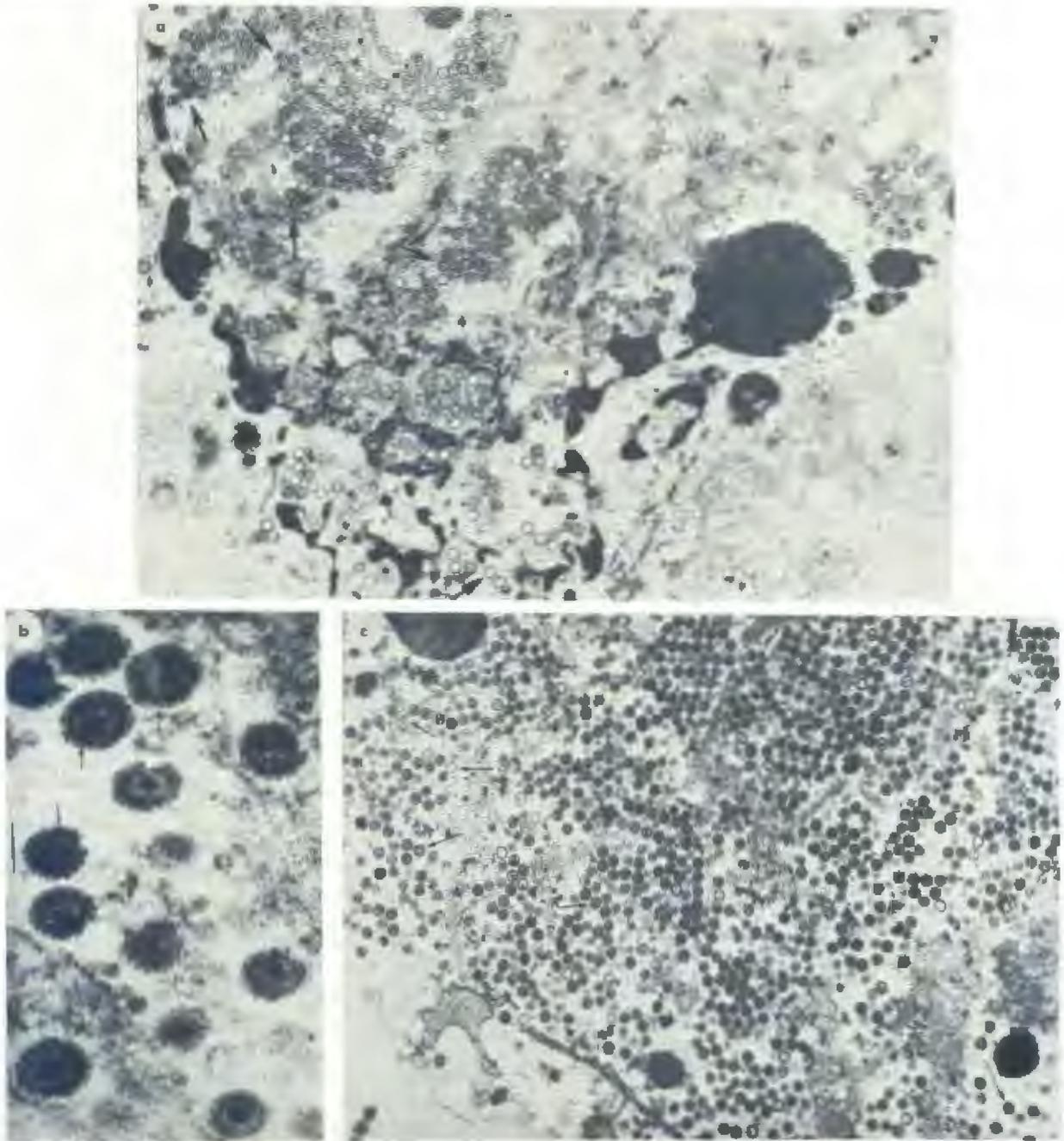


FIG. 5 a. Developmental stages of H2/4 in the nucleus of FCK cells. Notice "pin wheel core" formation (middle arrow). Magn. 20×10^3
 b. H2/4 virions in FCK cells showing spikes on the envelope. Magn. 35×10^3
 c. H2/4 nucleocapsids and empty capsids packed in the nucleus. Magn. 20×10^3

Haematoxylin-eosin staining

The viruses used for this investigation were of the lowest possible passage level prior to plaque purification. MDBK cells were cultured in roller tubes and disposable Petri dishes. A staining method, slightly modified from that described by Paul (1973), was applied to monolayers removed from the surface of the glass and plastic by colodion as described by Theodoridis (1973).

Electron microscopy

Cultures of FCK cells infected with virus strains H2/2, H5/1, H8/1 and H11/4 were examined by electron microscopy. The cells from culture tubes showing advanced CPE were detached from the glass with a long needle and the resulting suspensions were centrifuged for 4 minutes at $1\ 000 \times g$. The pelleted cells were pre-fixed in 3% glutaraldehyde for 1 hour and post-fixed in 1%

osmium tetroxide, as described by Lecatsas & Weiss (1968). All viruses included in this study were also examined by the negative staining method of Almeida & Howatson (1963). In this case, pelleted cells were disrupted with distilled water and stained with 3% phosphotungstic acid at pH 6. A drop of this mixture was transferred to a formvar-carbon-coated grid and the excessive fluid was drained with blotting paper. After air-drying, the grid was examined in an Elmiskope IA electron microscope.

Determination of nucleic acid

The type of nucleic acid was determined by the method of Hahnefeld & Hahnefeld (1964), using 5-iodo-2-deoxy uridine (IUDR). Cell cultures were treated with $30 \mu\text{g}/\text{ml}$ of IUDR in Eagle's medium for 24 hours prior to infection. Medium containing the same concentration

TABLE 4 Effect of chloroform, diethyl ether and trypsin on BHV-1 and BHV-4 viruses

Serogroup	Strain of virus	Untreated	Chloroform		Diethyl ether		Trypsin	
			Treated	Log inhibition	Treated	Log inhibition	Treated	Log inhibition
BHV-1	H1/3	5,0*	0,5	5,0	0,5	5,0	0,5	5,0
BHV-1	H11/4	6,0	0,5	6,0	0,5	6,0	0,5	7,0
BHV-4	H2/4	7,0	0,5	7,0	0,5	7,0	2,5	4,5
BHV-4	H3/4	6,5	0,5	6,5	0,5	6,5	2,0	4,5
BHV-4	H4/4	7,0	0,5	7,0	0,5	7,0	2,0	5,0
BHV-4	H5/1	6,5	0,5	6,5	1,0	5,5	2,5	4,0
BHV-4	H7/2	7,5	0,5	7,5	5,5	2,0	2,0	5,0
BHV-4	H8/1	6,5	0,5	6,5	0,5	6,5	2,0	4,5
BHV-4	H9/1	6,5	0,5	6,5	3,0	3,5	0,5	6,5
BHV-4	H10/1	5,5	0,5	5,5	2,0	3,5	2,5	3,0
BHV-4	H11/1	6,5	0,5	6,5	4,0	2,5	3,0	3,5
BHV-4	H12/1	7,5	0,5	7,5	0,5	7,5	2,5	5,0
BHV-4	H14/1	6,5	0,5	6,5	1,0	5,5	3,5	3,0
BHV-4	H15/1	6,5	0,5	6,5	0,5	6,5	3,0	3,5
Controls	ECBO	0,5	6,5	6,5	6,5	0,5	NT	NT
	EH2	5,0	NT	NT	NT	NT	0,5	5,0
	Cytomegalo	2,0	NT	NT	NT	NT	2,0	0,5

NT = Not tested

* Log to base 10 TCID₅₀/ml

TABLE 5 Cross-neutralization reactions between BHV-1 and BHV-4 viruses using specific goat antisera

Antigens	Antisera													
	H11/4	H1/3	H2/4	H3/4	H4/4	H5/1	H7/2	H8/1	H9/1	H10/2	H11/6	H12/1	H14/1	H15/1
Ref. strain H11/4	3,9*	3,5	—	—	—	—	—	—	—	—	—	—	—	—
Isolates H1/3	3,5	4,5	—	—	—	—	—	—	—	—	—	—	—	—
Isolates H2/4	—	—	4,0	2,2	3,1	3,2	3,2	2,0	3,7	2,5	1,8	5,3	3,7	2,8
Isolates H3/4	—	—	1,8	2,0	2,8	1,8	1,8	1,8	1,8	1,8	1,8	—	3,3	2,0
Isolates H4/4	—	—	3,3	2,0	2,0	3,3	4,5	2,5	—	—	1,8	4,5	4,5	4,0
Isolates H5/1	—	—	4,5	4,0	4,5	5,0	5,0	5,0	3,5	4,5	4,5	3,5	5,0	3,0
Isolates H7/2	1,8	2,0	2,7	1,8	1,8	2,0	5,7	2,2	—	—	2,2	2,2	2,2	1,8
Isolates H8/1	—	—	2,5	2,5	2,5	2,5	1,8	2,5	1,8	1,8	1,8	2,5	2,5	1,8
Isolates H9/1	—	1,8	3,5	5,6	3,5	4,7	4,7	4,7	4,5	3,5	5,6	2,0	4,7	3,1
Isolates H10/2	—	—	3,0	4,0	4,0	4,5	4,5	3,2	3,2	3,2	5,5	2,0	4,5	3,0
Isolates H11/6	—	1,8	5,0	2,8	3,3	3,8	1,8	5,3	—	1,8	2,8	3,8	5,3	2,3
Isolates H12/1	—	—	4,0	1,8	2,0	2,5	1,8	2,5	1,8	1,8	1,8	2,5	4,0	2,5
Isolates H14/1	—	1,8	—	2,5	2,1	2,3	2,0	2,4	1,8	3,0	2,5	2,7	5,5	1,8
Isolates H15/1	—	—	3,0	1,8	1,8	1,8	1,8	—	1,8	1,8	—	—	—	1,8

* Log to base 10 TCID₅₀/ml

— Log of neutralizing index less than 1,8

TABLE 6 Cross-complement fixation reactions between BHV-1 and BHV-4 viruses with specific goat antisera

Antigens	Antisera													
	H11/4	H1/3	H2/4	H3/4	H4/4	H5/1	H7/2	H8/1	H9/1	H10/2	H11/6	H12/1	H14/1	H15/1
Ref. antigen H11/4	8*	4	<4	<4	<4	16	32	4	16	8	16	4	8	16
New isolates H1/3	4	4	8	128	<4	8	16	8	16	8	32	16	4	32
H2/4	4	<4	<4	32	<4	4	32	16	32	32	32	8	8	32
H3/4	<4	<4	<4	128	<4	32	32	32	32	32	8	8	8	32
H4/4	8	8	<4	16	8	64	128	32	64	32	32	16	16	64
H5/1	4	<4	<4	<4	<4	16	16	4	4	<4	8	<4	<4	32
H7/2	<4	<4	16	4	<4	16	64	8	128	4	32	8	8	32
H8/1	8	4	<4	<4	32	4	4	16	4	4	<4	16	16	<4
H9/1	8	8	<4	128	<4	64	128	32	128	32	32	4	4	32
H10/2	4	4	16	<4	<4	16	16	8	8	16	16	8	4	32
H11/6	4	8	<4	8	<4	16	16	8	8	8	32	8	<4	32
H12/1	8	8	8	8	4	16	32	8	8	16	32	8	8	32
H14/1	8	8	<4	8	4	32	128	32	64	32	32	8	16	32
H15/1	8	<4	<4	8	<4	16	16	<4	8	<4	32	8	4	32

* Reciprocal of twofold serum dilution exhibiting 75 % or more fixation of complement

of IUDR was again added to the cultures after each had been infected with 0,2 ml of 1:10 dilution of virus. Adsorption proceeded for 1 hour at 37 °C. Untreated control cultures were infected with the same virus concentration. When CPE involved 50 % or more of the cells in the untreated cultures, the virus yield in both treated and untreated cell cultures was assayed.

Physicochemical properties

Sensitivity to chloroform: Virus suspensions harvested from FCK cell cultures with advanced CPE were centrifuged at 4 000 × g for 90 minutes and the supernatant

fluid was removed for the test. Sensitivity to chloroform was determined according to the method described by Bögel & Mayr (1961). The untreated and treated virus suspensions were titrated in FCK cells. In both the above tests a strain of enteric cytopathic bovine orphan (ECBO) virus was included as an additional control, being a known RNA-containing, chloroform-stable virus (Oelermann, Els & Verwoerd, 1967).

Sensitivity to diethyl ether: A freshly prepared virus suspension was centrifuged at 4 000 r.p.m. for 30 minutes and to the supernatant fluid diethyl ether was

added in an amount equal to 20 % by volume. The bottle was tightly closed and the contents mixed well by shaking, after which it was placed for 20 hours at 4 °C. The treated suspension was placed in a Petri dish to allow the ether to evaporate. Both treated and control virus suspensions were titrated in roller tube cell cultures as previously described.

Effect of trypsin: A 1 % trypsin solution (Difco) was mixed with an equal volume of virus suspension free of cell debris. The mixture and the control samples consisting of virus and buffer were held for 1 hour at 32 °C. The trypsin activity was stopped by adding 4 volumes of antibody-free bovine serum. The treated and control samples were titrated in FCK cells in roller tubes and the infectivity titres were determined according to the development of CPE on the 8th day.

Equid Herpesvirus 2 (EH2) and an equine cytomegalovirus were included as known sensitive and resistant strains (Erasmus, 1970).

RESULTS

Development of cytopathic effect and replication of the virus

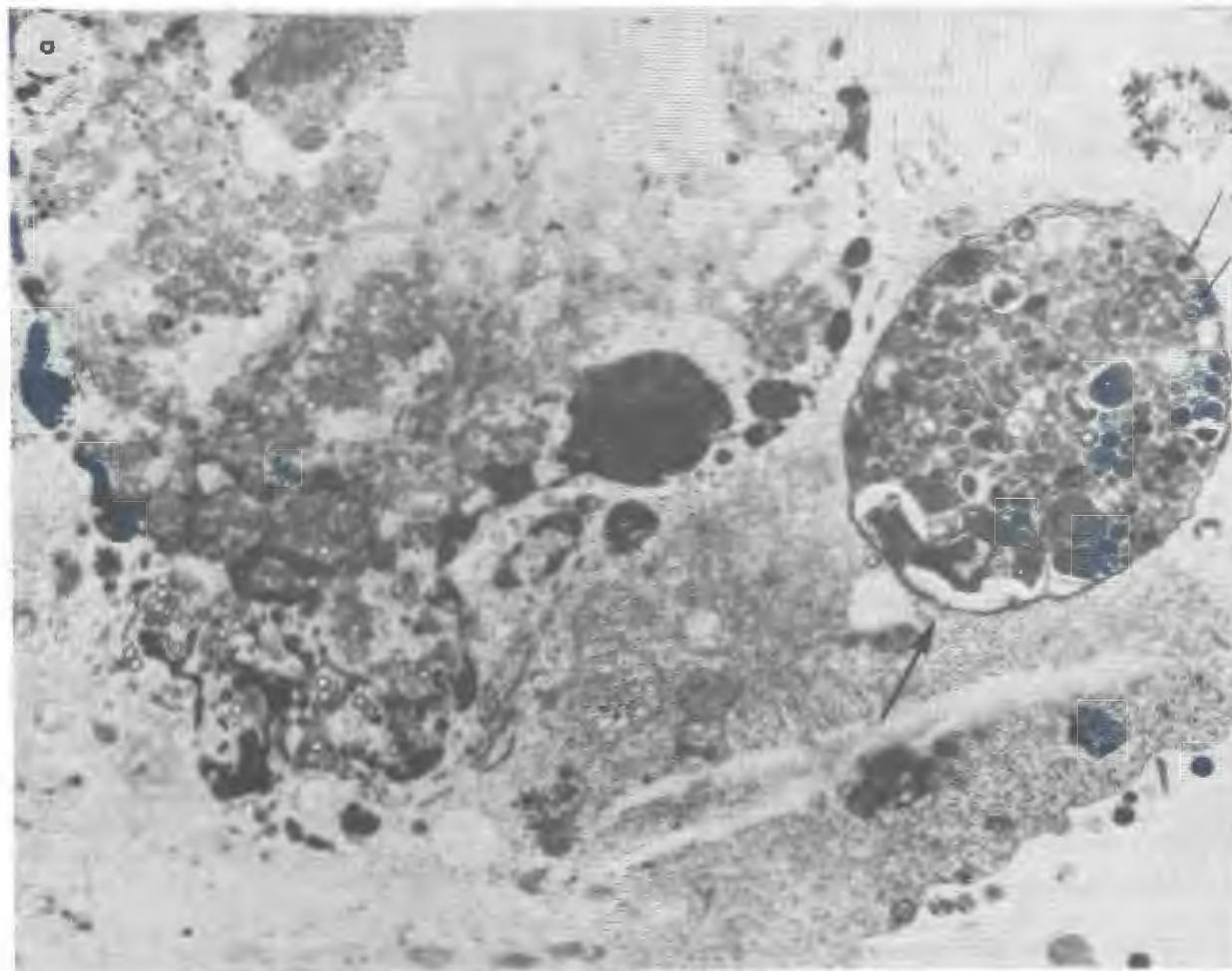
A detailed study of the CPE of the plaque-purified viruses was undertaken in FCK line cells. According to the rate of development of CPE at an initial infectivity of 10^3 TCID₅₀/ml, the viruses could be divided into 2 groups. The group with the shortest replication cycle included the known IBR-IPV virus H11/4 derived from isolate FH335 and strains H1/3 and H13/1, while the group with the extended replication cycle consisted of H2/4, H3/4, H4/4, H5/1, H7/2, H8/1, H9/1, H10/2, H12/1, H14/1 and H15/1. At this stage it was decided to

follow the designation of McKercher (1973) and Gibbs & Rweyemamu (1977) whereby isolates H11/4, H1/3 and H13/1 will be referred to as BHV-1 and the remainder as BHV-4 viruses.

In the case of the slow growing viruses (BHV-4), CPE was first noticeable on the 3rd day after infection in the MDBK cells at the periphery of the culture. This process extended slowly to involve the entire culture. Complete destruction of all the cells usually took up to 10 days. The affected cells appeared rounded, shrunken and less translucent and formed irregularly-shaped groups (Fig. 1a). The cells did not become detached immediately and no empty spaces in the monolayer were visible during the early stages, despite destruction of the cells at the initial foci (Fig. 1a). Cell debris was noticed from the 3rd to 4th day after inoculation and the media became cloudy as the CPE advanced. The shrinking of the infected cells was slower than those infected with BHV-1, although cell debris was noticeable prior to the formation of foci or the appearance of open spaces (Fig. 1b). Cells contained intranuclear inclusions in areas where CPE could not be seen with the light microscope (Fig. 1c). Orientation of cells towards foci was clearly shown in FCK cultures infected with the BHV-4 viruses (Fig. 1d).

The fast growing viruses (BHV-1) formed foci of rounded cells throughout the culture from the 2nd day of inoculation and open spaces were observed in the centre of the foci at the first appearance of CPE (Fig. 2e & f). Cells appeared rounded, refractile and pycnotic right from the start when CPE was first noticed. The entire monolayer was affected after the 3rd or 4th day.

With the exception of virus H8/1, which failed to produce CPE in BHK21 cells, all the remaining viruses



produced CPE in FCK, FCL, FCT, BHK21, VERO and MDBK cultures. The nature of the CPE and the rate of development varied according to the cell type, the most remarkable being the VERO cells where CPE never exceeded 50 % of the total culture, even after prolonged incubation. Although H8/1 did not produce CPE in BHK21 cells, infected cultures harvested on the 8th day had an infectivity titre of 10^6 TCID₅₀/ml when assayed in FCK cells. All the virus strains produced plaques in FCK cell monolayers. The strains of BHV-4 produced very small plaques, approximately 1–2 mm in diameter, which did not increase in size during the 8-day incubation period. They remained compact, granular and well-demarcated. The plaques produced by the BHV-1 viruses were larger, measuring 4–5 mm in diameter, with rounded translucent cells on the edges. The size of each plaque increased with continued incubation.

Despite the fact that the strains of BHV-4 viruses grew well in 2 cell types (FCK and MDBK), the selection of the most susceptible cell was necessary for further studies. In this respect the morphology of the plaque, the speed of development of CPE and the immunogenicity of the viral suspension were some of the parameters considered. As was previously indicated, complete cell destruction in MDBK cultures could take as long as 10 days. This period was shortened to 6 days in FCK cells. In FCL cells, on the other hand, it was found that CPE started between 36 and 48 hours and the cultures were destroyed within 72 hours after inoculation. Likewise, the development of the CPE of H11/4 was also shortened in these cells, and, commencing 24 hours after infection,

it reached 100 % within 36 hours. The virus preparations from these cultures were used for electron microscopy, the production of immune sera in goats and infection of heifers in the pathogenicity studies.

Variation in CPE amongst the strains of BHV-4 viruses was difficult to evaluate except in 1 instance when the strains were cultured in MDBK cells at 32 °C in stationary Roux flasks. Under these conditions, they could be separated into 4 groups on the basis of the appearance of the CPE:

Group A—H2/4, H5/1, H7/2, H8/1, H11/6, H14/1, H15/1.

Group B—H4/4, H10/2 and H12/1.

Group C—H9/1.

Group D—H1/3 and H11/4.

In Group A the CPE commenced along the edges where the cells appeared less translucent and shrunken, but not necessarily rounded. Only small groups of affected cells could be observed without any particular morphological features. On the 3rd day, single cell lesions could be observed throughout the culture. The affected cells maintained their original shape until they were detached from the glass. The CPE increased by spreading throughout the culture and involving most of the cells. Only a small percentage were unaffected and these remained attached to the glass until the 8th day.

The Group B viruses produced large, dense, round foci, dispersed throughout the culture. Very few single cell lesions appeared to be present. The cells in the foci

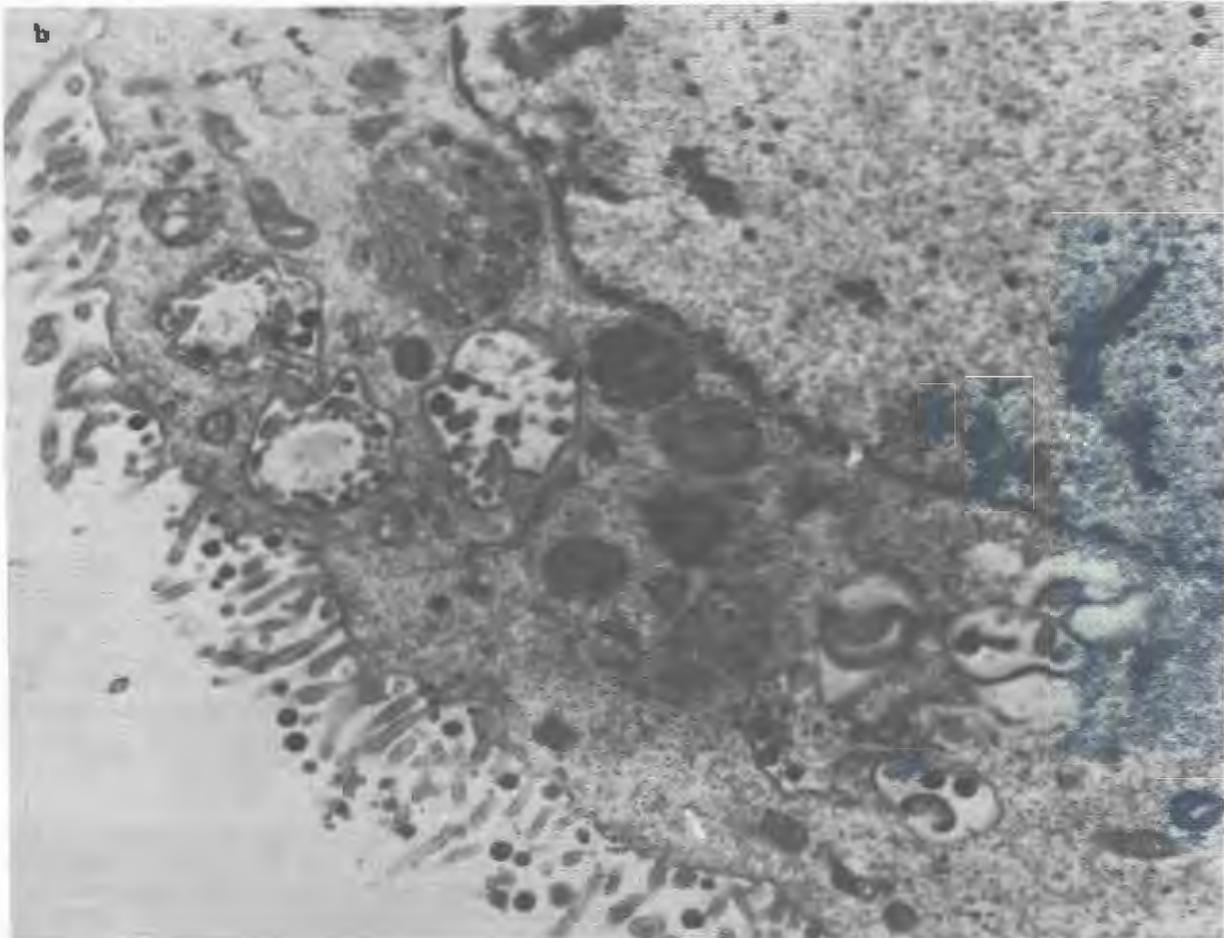


FIG. 6 a. Matrix in cytoplasm of FCK cells infected with H2/4. Magn. 20×10^3
 b. Pseudopodia formation in FCK cells infected with H2/4 virus. Magn. 20×10^3

were elongated and appeared interwoven, thus giving the impression that the foci were denser than the rest of the culture. On the 5th day, all the cells were affected and many were detached. Those remaining represented the original foci.

In Group C, which consisted of 1 strain only, rounded cells were seen dispersed homogeneously throughout the culture, with some grouping of rounded cells, although many open spaces could also be seen. The CPE appeared very similar to the cell changes caused by BHV-1 virus. By the 5th day, CPE had involved 100 % of the cells.

The CPE of Group D was the same as that described earlier for the BHV-1 viruses, which included the IBR-IPV virus.

The BHV-1 viruses incubated at 32 °C produce 3 types of CPE in MDBK cells, while the viruses incubated at 37 °C produce only one type of CPE in MDBK cells. The above observations show how the CPE of herpesviruses can vary according to the cell type and the temperature at which they are cultured. These observations, however, were reproducible in roller tubes.

Haematoxylin-eosin staining of infected cells

In this experiment, 12 strains of virus representing 10 BHV-4 and 2 BHV-1 viruses were examined. All the strains produced intranuclear inclusion bodies of Cowdry type A (Fig. 1, b, c & d). There were no differences in the type, size and staining of the inclusion bodies between the 2 groups of strains. In the cultures infected with the BHV-1 strains, the inclusion bodies could be seen in cells situated mainly around the margin of the CPE focus, while in the cultures of the BHV-4 strains they were evenly distributed throughout the culture in addition to being present around the foci of CPE. It was of interest to note that the CPE of the strain H9 was only 20 % on the 4th day, although 75 % of the cells showed inclusion bodies. All the strains of BHV-4 showed a similar pattern of distribution of inclusion bodies, but of lower density. The nuclei and cytoplasm of many infected cells were enlarged (giant cells) and an early cellular destruction was visible in the centre of the foci which appeared multilayered. The surrounding cells appeared to be oriented towards the centre of the focus as if there was a migration towards it. No syncytia were seen in the cultures at any time.

Growth curve

The sample of cells which was taken 2 hours after infection contained infective virus at a concentration of 3×10^2 TCID₅₀/ml (Fig. 2). There was no detectable infective virus in the cells between the 4th and 14th hour of incubation. However, infective virus was again demonstrated at 16 hours and thereafter a sharp increase was observed. The highest concentration of virus was reached at 46 hours and was maintained until 54 hours when sampling was discontinued. At this stage, all the cells showed CPE, the majority of them being detached from the glass.

In the cell-free medium, virus was demonstrated at 24 hours. The increase in virus concentration followed the same pattern as that found in the cells, but it was of lower titre. After 24 hours the concentration was comparable in both the cells and medium. Here again the highest concentration of virus in the medium was found at 46 hours, but it was nevertheless slightly lower than the concentration in the cells.

Influence of temperature on the envelopment of BHV-4 viruses

Pelleted virus of strains H2/4 and H14/1, incubated at 37 °C in FCK and MDBK cells, consisted of almost 100 % nucleocapsids (Fig. 3a) while strain H11/4, grown

under the same conditions, showed 30 to 40 % enveloped particles and 60–70 % nucleocapsids (Fig. 3b). As the incubation temperature decreased to 34 °C and 32 °C, the percentage of enveloped particles of H2/4 strain increased (Fig. 3c and d). At 32 °C, in FCK cells, the negative-stained enveloped particles showed a pleat-like structure, resembling a tail which appeared to be formed by the loose envelope (Fig. 3d). This structure was also observed in the preparations obtained at higher temperatures, but to a much lesser extent.

H2/4, cultivated in FCL cells, consisted of almost 80 % enveloped particles at all 3 temperatures of incubation (Fig. 4a), and the loose envelope was visible in almost all particles.

In sections of cells infected with H2/4 and incubated at 32 °C one could see what appeared to be a predominance of enveloped particles in the cytoplasm. In the same preparation, that the space between virus capsid and envelope was stained suggested the existence of electron dense material (Fig. 4b). The above detail, however, was inconsistent and present to a lesser extent in FCL cells infected with H11/4 virus and incubated at low temperatures (Fig. 4c).

Effect of protamine sulphate

In an attempt to differentiate between the different viruses, the effect of protamine sulphate on plaque formation was investigated. It appeared that there was no clear pattern regarding the effect of protamine sulphate on plaque size and number. As Table 2 showed it would appear that the plaque-forming unit titre of some viruses had increased, while with others it had decreased. On the other hand, the plaque size of H3/4, H5/1, H7/2, H8/1, H9/1, H10/2, H12/1 and H15/1 almost doubled in the treated cultures, while H2/4, H11/6 and H14/1 remained unchanged (Table 2). There was thus no correlation between the effect of protamine and the tentative grouping of the strains.

Nucleic acid determination

There was more than 90 % reduction in the concentration of virus produced in cultures treated with IUDR (Table 3). This result proved beyond doubt that all the BHV-1 and BHV-4 viruses contain DNA as genome. There was no change in the concentration of the ECBO virus used as a control in the test.

Effect of chloroform

The effect of chloroform on the infectivity of these herpesviruses is shown in Table 4. Strains of BHV-1 and BHV-4 viruses were completely inactivated by the chloroform treatment. There was no effect on the control virus represented by an ECBO virus resistant to lipid solvents.

Effect of diethyl ether

The BHV-1 strains were totally inactivated when treated with diethyl ether (Table 4). Of the BHV-4 viruses, some were totally inactivated, while others showed a degree of resistance, with residual titres between 1 and 5.5 (Table 4). This variation of sensitivity to diethyl ether is not in accordance with the antigenic relatedness or other characteristics of these viruses.

Effect of trypsin

Trypsin completely inhibited the infectivity of both BHV-1 viruses (H1/3 and H11/4) and of one of the BHV-4 viruses (H9/1) (Table 4). The infective titres of the remaining viruses of Group 11 were reduced to a lesser extent. The EH2 control, which is sensitive to trypsin, was inactivated, while the infectivity of the equine cytomegalovirus remained unchanged.

Electron microscopy

The examination under the electron microscope of cells infected with strains H2/3, H5/1, H8/1 as representatives of BHV-4 and H11/4 of the BHV-1 group, revealed a typical herpesvirus morphology. Unenveloped hexagonal particles, approximately 100 nm in size, could be seen in the nuclei of the cells. Other developmental stages, such as unenveloped empty capsids, some with rod-shaped cores and others with large dense cores as illustrated in Fig. 5a, were also seen. Enveloped particles, approximately 140 nm in size, were found in the cytoplasm (Fig. 5b). Structures observed on the enveloped virions resembled spikes (Fig. 5b).

All the strains of virus examined after preparation by the negative contrast staining method confirmed the typical morphology of the family Herpesviridae (Fig. 3a & 3b).

Strain H2/4, incubated in FCK cells at 37 °C, showed a marked accumulation of particles in the nucleus, with very few particles in the cytoplasm (Fig. 5a & c). A large number of particles were seen with granules in the nucleocapsids, while in other particles a large core was visible (Fig. 5a). Large conglomerations of unenveloped particles were observed in what appeared to be a movement into the cytoplasm where the nuclear membrane was disintegrated (Fig. 5c). With H2/4 and other strains of BHV-4, large pockets of heterogeneous particles were frequently seen in the cytoplasm (Fig. 6a). Occasionally, FCK cells infected with the BHV-4 strains showed pseudopodia (Fig. 6b).

Neutralization test

The BHV-1 isolates (H11/4 and H1/3) produced antibodies in rabbits, goats and cattle and neutralized at least 5 logs of homologous virus. The BHV-4 isolates failed to induce the formation of neutralizing antibodies in rabbits and cattle, and very low neutralizing antibody concentrations were detected in goats after the first attempt at immunization with virus cultured in FCK and MDBK cells. The goat that received purified virus (H14/1) from infected FCL cells failed to develop neutralizing antibodies, while the goat that received an unpurified suspension of virus did in fact develop a detectable antibody concentration. After this trial, when another group of goats was injected with virus prepared in FCL cells, all of them developed satisfactory antibody titres.

The influence of complement on the neutralization reaction was investigated with strain H2/4 and its homologous serum. The neutralization index of the treated antiserum was higher by 0.7 log than in the untreated serum. In 2 tests, conducted in different cell types, it was shown that this difference was consistent.

The results of the cross-neutralization tests with the goat sera carried out in FCK and MDBK cells are presented in Table 5. These results indicate that the viruses belong to 2 distinct serological groups representing BHV-1 and BHV-4 serogroups. There appears to be a complex inter-relationship among the BHV-4 strains as well as between them and the BHV-1 strains. With some of the BHV-4 strains, the neutralization index against the heterologous sera was higher than with the homologous sera (H2/4, H3/4, H9/1, H10/2, H11/6, H12/1 and H15/1). Among the BHV-1 strains, H2/4 antiserum neutralized all 12 strains and the virus was neutralized by all antisera with the exception of H14/1. Other strains of viruses were neutralized by fewer antisera, while their specific antisera neutralized a greater variety of antigenic variants (H12/1). The antisera of H9/1 and H10/2 neutralized only 5 strains each, while the viruses were neutralized by all 12 antisera. The poorest inter-relationship was shown by H15/1 and H7/2. The antiserum of BHV-1

strain H11/4 nevertheless weakly neutralized the viruses H7/2, H9/1, H11/6 and H14/1. H7/2 virus was also slightly neutralized by the antiserum of H1/3. The antisera of all the BHV-4 viruses failed to neutralize the BHV-1 viruses (Table 5).

Complement fixation test

The results of this test showed that the goats experimentally inoculated with the herpesviruses developed complement fixing antibodies (Table 6). Viral antigens, prepared in FCL cell cultures maintained in Eagle's medium without serum and lactalbumin, provided satisfactory antigens for this test.

The H11/4 strain of BHV-1 showed fixation of complement with the antisera of 6 strains of BHV-4 virus. Although there was no clear pattern of cross-reaction, the BHV-4 strains showed either direct or indirect relationships with each other. H3/4 for example was related to H2/4 and H4/4, but all 3 were related to H5/1. Despite these relationships, there is no clear division of these viruses into antigenic groups, however.

The sera used in this test were produced in hyper-immunized goats, and unfortunately the development of CF antibodies in these animals was not followed up to establish the optimal time at which CF antibodies were at their highest concentration. This shortcoming may be the reason for the complex picture created by the results of this test.

Although the CF test results are not directly comparable with those of the neutralization test, some viruses which showed poor antigenic relationship with other strains in the neutralization test showed the opposite effect in the CF test. The cross-reactions between H15/1 and H12/1 are examples of this effect.

DISCUSSION

The first distinguishing characteristic of the 12 BHV-4 strains was obtained from electron microscopy. The morphology of the particles seen in thin sectioned cells was identical with the detailed description of herpesviruses given by Morgan, Ellison, Rose & Moore (1953). In the negatively stained samples, the particles resembled herpesviruses in both shape and size, as described by Brenner & Horne (1959). The morphology and size of the observed particles coincided with the basic forms described by Wildy *et al.* (1960). However, during the course of the experiments with the BHV-4 viruses, it was noticed that, at higher passage levels in MDBK cells at 37 °C, only nucleocapsids were observed in the culture media. Surprisingly, there was no reduction of the virus titres at these or successive passage levels or when further passes were made in FCK and FCL cells cultured in roller tubes or Roux flasks. This observation suggests that nucleocapsids produced *in vitro* are as infective as enveloped particles.

The predominance of naked nucleocapsids in MDBK and FCK cells could be a result of the infected cells being disrupted before the normal process of virus maturation and release can take place. The information acquired while studying the development of CPE supports this hypothesis. During the early stages of CPE, on the 3rd or 4th day, cell debris was observed in the media, which gradually became cloudy. In other BHV-4 strains, clumps of rounded cells was the predominant CPE seen (Ludwig, 1983). In contradistinction, BHV-1 virus-infected cultures did not show rupture of cells, but rather rounding up, cell clustering, shrinking and finally detachment from the glass wall. As an exception to the rule, H2/4 produced almost entirely enveloped particles when FCL cells were infected and incubated at 37 °C.

It is evident that cell fusion and syncytia formation are not the only ways in which nucleocapsids spread from cell to cell, and that a normal infection via the free cell surface can take place. In the *in vivo* situation, the method of viral spread from cell to cell is unknown. Cell fusion is a characteristic of BHV-2 (bovine mammillitis), but not of BHV-1 and BHV-4.

Plaque formation by BHV-1 and BHV-4 strains in this study was very similar to that reported by Ludwig (1983). All our BHV-4 strains formed plaques of 1 mm diameter in about 8 days, while the BHV-1 strains formed larger plaques within 2–3 days (Theodoridis, unpublished results, 1980). The growth curve of the H2/4 strain in FCL cells confirmed the slow growth which is characteristic of other BHV-4 strains (Ludwig, 1983). Considering the fact that CPE develops much faster in FCL cells than in MDBK cells, growth in the latter can be expected to be even slower (Theodoridis, 1978).

The influence of temperature on the production of enveloped virus particles was investigated. It was shown that for the BHV-4 viruses the highest concentration of enveloped particles was detected in MDBK cultures incubated at 34 °C, while in FCL cells 90 % were enveloped at this temperature. The proportion of nucleocapsids to enveloped particles in MDBK cells at 32 °C was 70 % to 30 %. The majority of the envelopes were loosely arranged around the nucleocapsid and, when centrifuged, they formed a pleat which appeared like a tail. The same phenomenon has also been observed with BHV-1 viruses as well as with other herpesviruses (Wildy *et al.*, 1960).

The variation in the CPE observed in the various cell types was characteristic of the cell itself. In the MDBK cells of the 200th passage, all BHV-4 strains started growing on the edges of the culture and progressed slowly to the central area of the monolayer by the 10th day. In the FCK or FCL cell subcultures, the CPE was evenly distributed from an early stage and it destroyed the culture by the 3rd to 4th day under conditions of comparable infection. However, the difference in the rate of the development of CPE between the BHV-1 and BHV-4 viruses remained the same in different cell types.

Sabina & Parker (1963) emphasized the importance of the quality of the cells in the growth of IBR virus (BHV-1). The replication of this group of viruses would appear to be very dependent on the biophysical state of the cell and the temperature of incubation.

Rabbits, cattle and goats failed to produce specific antibodies against the BHV-4 viruses cultivated in FCK and MDBK cells. In addition, purified virus of strain H14/1 prepared from FCL cells failed to induce the production of neutralizing antibodies in a goat. In contrast, the BHV-1 strains (H11/4 and H1/3) produced very potent and specific antisera in all these host systems. However, when cell cultures (FCL) infected with the BHV-4 viruses were suspended in oil adjuvant and injected into goats, neutralizing and CF antibodies to homologous and heterologous strains were produced.

Ashe & Sherp (1965) demonstrated antigenic variation in strains of herpes simplex virus isolated from successive recurrences of herpes labialis, by comparing the kinetics of the homologous and heterologous neutralization reaction. It is possible that the herpesviruses of cattle may show similar antigenic variation. However, the neutralizing antibodies against the BHV-4 viruses were too low in concentration to permit such a distinction being made by the same method. Only a weak, one-way neutralization was found between the BHV-1 and BHV-4 groups, whereas a complex inter-relationship within the BHV-4 group was revealed.

A difference in the efficiency of neutralization between the 2 groups was also found. The BHV-4 viruses would break through at 8–10 days with loss of inhibition, whereas the BHV-1 viruses failed to show this characteristic. Efficient antisera against the latter would neutralize 10^5 TCID₅₀/ml of virus without ever breaking through, even after 10 days. To make it possible to evaluate the inhibiting effect on BHV-4 viruses, the inoculated cultures had to be examined daily. The poor reactivity of the neutralization antibodies against the BHV-4 viruses can possibly be attributed to the configuration of the reactive sites. It is possible that the antibodies react with "non-critical" antigenic sites which may sterically hinder the binding of other antibodies to the "critical" sites and thus result in free unneutralized particles. This explanation has been proposed in the case of herpes simplex virus by Yoshino & Morishima (1972), for a phenomenon similar to that seen in the case of BHV-4 viruses. Very few foci appear in the early stages after neutralization, but the antibodies present cannot completely neutralize newly released particles, and this results in delayed CPE or breakthrough.

The effect of a co-factor-like complement on neutralization, described by Stevens, Pincus, Burroughs & Hampar (1968) and Hampar, Stevens, Martos, Ablashi, Burroughs & Wells (1969), was not observed in the case of the bovine herpesviruses studied. Varying the incubation time of the antigen-antibody binding reaction at 37 °C or 4 °C was also without effect on the neutralization test.

Potgieter & Maré (1974) succeeded in demonstrating neutralizing antibodies to herpesviruses from the respiratory tract of cattle by determining the kinetics of the reaction. These viruses were previously also thought to produce no neutralizing antibodies (Mohanty, Hammond & Lillie, 1971). The neutralization of these viruses might have been more significant if antibody and complement had been added at short intervals. Babiuk, Wardley & Rouse (1975) demonstrated that IBR-infected cells were susceptible to antibody-complement lysis at 10 hours after infection. When antibody and complement were added at 2-hour intervals, the spreading of virus from cell to cell could be arrested.

As was previously mentioned, the initial electron micrographs revealed that all the isolates belonged to the herpesvirus group. This classification was provisionally accepted and later supported by other tests. Further EM studies of some of the strains raised certain problems which required additional explanation. Sections of cells (FCL) infected with BHV-4 viruses and cultured at 32 °C presented a peculiar particle. The space between the capsid and envelope was filled with what appeared to be an electron-dense layer. Roizman, Spring & Schwartz (1969) proved that this layer in the herpesviruses is not resolved in the electron microscope and that it consists of some type of protein which keeps the envelope at a certain distance from the core and may be the 2nd envelope that makes naked particles infective. The outer envelope of the herpesviruses included in this study showed projections which corresponded to the spikes that are characteristic also for herpesviruses (Morgan *et al.*, 1954, Watson & Wildy, 1963). In sections of infected CFK cells cultivated at 37 °C, the "pin wheel core" phenomenon was also observed.

Similar granules have been reported in avian herpesvirus by Watrach, Hanson & Watrach (1968), in turkey herpesvirus (Nazerian, Lee, Witter & Burmeister, 1971), in equine abortion virus (O'Callaghan, Hyde, Gentry & Randall, 1968), in herpes simplex virus (Friedman, Coward, Rosenkranz & Morgan, 1975) and in the tree shrew virus (McCombs, Brunschwig, Mirkovic &

Benyesh-Melnick, 1971). Miyamoto & Morgan (1971) suggested that they were the precursors of the hollow core of herpes simplex virus. As with the herpesvirus particles of various species, the viruses in this study appeared in the cytoplasm in groups or singly, surrounded by a cellular membrane. The commonly encountered form of the BHV-4 strains is shown in Fig. 5 & 6. It consists of a conglomeration of heterogeneous particles, surrounded by a cellular membrane. Very few free particles were present in the cytoplasm. The cell surface of FCK cells showed pseudopodial activity which was not seen with the BHV-1 infected cells or control cells examined at the same time.

In Fig. 5c, a possible explanation is given for the earlier observation that the majority of particles produced at 37 °C are nucleocapsids. The nucleocapsids seem to be in the process of liberation as they are half-way into the cytoplasm, and the nuclear membrane appears broken.

Alternatively, if the preformed nucleocapsids for some reason do not move into the cytoplasmic vacuoles, but accumulate in the nucleus, they would not get the chance of acquiring an envelope. The situation is different when the incubation takes place at a temperature of 34 °C or lower. Under these conditions many enveloped particles appeared in the cytoplasm and the nucleus was not overloaded with nucleocapsids (Fig. 4b & c). This would appear to represent the optimal conditions for viral replication.

Basing our classification on the biological, morphological and physicochemical characteristics discussed above, we provisionally put all the isolates except H1/3 in the bovid herpesvirus-4 (BHV-4) group proposed by Ludwig (1983). H1/3 belongs to the BHV-1 group. It is possible that the BHV-4 isolates may be one and the same virus which has undergone certain biological changes, which is typical for herpesviruses.

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