

## PLAQUE FORMATION BY AFRICAN HORSESICKNESS VIRUS AND CHARACTERIZATION OF ITS RNA

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### ABSTRACT

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A method of plaque assay for African horsesickness virus (AHSV) is described. Three cell lines were tested of which BHK21 was the most satisfactory.

With an increase in Actinomycin D concentration, an increased inhibition of infective virus production was observed.

Sucrose gradient sedimentation analysis of AHSV-RNA revealed at least six components. Eight components were resolved by polyacrylamide gel electrophoresis. The molecular weight of the components varied from  $0.5 \times 10^6$  to  $2.8 \times 10^6$  daltons, with a total molecular weight estimate of  $1.5 \times 10^7$  daltons for 10 segments in the viral genome. The significance of the relationship between AHSV, bluetongue virus and reovirus is discussed.

### INTRODUCTION

The production of plaques by African horsesickness virus (AHSV) on MS and VERO cells has been described by Mirchamsy & Taslimi (1966a), Hopkins, Hazrati & Ozawa (1966) and Hopkins, Ozawa & Hazrati (1967). The effect of different experimental variables on plaque formation was investigated and the period of incubation required for plaque formation on MS cells was, according to Mirchamsy & Taslimi (1966a), 5 to 12 days and, according to Hopkins *et al.* (1966), as long as 14 days. Plaque formation on VERO cells was only achieved after 15 to 19 days (Hopkins *et al.*, 1967). These extended periods of incubation are considered excessive for the plaque assay to be of any advantage over the TCID<sub>50</sub> for purposes of assay of AHSV.

Mirchamsy & Taslimi (1966b) and Oellermann, Els & Erasmus (1969), using MS and BHK21 cells respectively, observed a growth inhibitory effect on AHSV by Actinomycin D at concentrations varying between 0.05 and 0.5  $\mu\text{g/ml}$ . Ozawa (1967), however, reported that 1.8  $\mu\text{g}$  Actinomycin D had no effect on the growth of AHSV on VERO cells. The question arises as to whether this difference in sensitivity can be ascribed to the difference in cell lines used.

It has been established by Oellermann *et al.* (1969) that AHSV has a double-stranded RNA genome which has properties very similar to that of bluetongue virus (BTV) (Verwoerd, 1969; Verwoerd, Louw & Oellermann, 1970) and reovirus (Bellamy, Shapiro, August & Joklik, 1967; Shatkin, Sipe & Loh, 1968; Watanabe & Graham, 1967; Watanabe, Milward & Graham, 1968). Recent hybridization studies revealed no homology between reovirus Type 1 and AHSV Type 3 and only a small amount of hybridization was observed between BTV Type 10 and AHSV Type 3 (Verwoerd & Huismans, 1969). Electron microscopic studies of negatively stained virus showed that AHSV and BTV are indistinguishable. Both possess protein capsids consisting of a single layer of 32 capsomeres arranged in regular icosahedral symmetry to form a particle 55 nm in diameter (Els & Verwoerd, 1969; Oellermann *et al.*, 1969). The main difference between AHSV and BTV on the one hand and reoviruses on the other is in the morphological characteristics of the capsids, reovirus particles being 77 nm in diameter and possessing double-layered capsids with 92 capsomeres (Loh, Hohl & Soergel, 1965). The significance of the similarities and differences

in the properties of this interesting group of viruses remains to be elucidated.

In the present investigation plaque formation by AHSV on different cell lines, including VERO, was re-investigated using agarose as basic overlay. Data on the growth inhibitory effect of different concentrations of Actinomycin D on AHSV and additional information on the segmented nature of its double-stranded RNA genome are presented.

### MATERIALS AND METHODS

#### Cells

Three cell lines were used during this investigation: BHK 21 and L-strain mouse fibroblast cells (Verwoerd, Oellermann, Broekman & Weiss, 1967) and VERO cells (originally obtained from Dr. Fujita, National Institute of Animal Health, Tokyo). The media used and the method of culture of the cells are described elsewhere (Verwoerd *et al.*, 1967; Oellermann *et al.*, 1969).

#### Virus

AHSV Type 9 (strain 90/61) was used throughout this investigation (Oellermann *et al.*, 1969).

#### Buffers

Tris buffer was an aqueous 0.002M Tris-(hydroxymethyl)-methylamine solution at pH 8.8. When 0.02M and 0.2M tris were used, the pH was adjusted to pH 8.8 (Table 1).

Phosphate buffered saline (PBS), pH 7.0, was prepared according to the method of Dulbecco & Vogt (1954). A hundred-fold aqueous dilution of PBS, designated 1/100 PBS, has also been used (Table 1).

STE buffer, pH 7.4, consisted of 0.1M sodium chloride in 0.005M tris and 0.001M ethylene diamine tetra acetic acid (EDTA).

#### Virus assay

##### (a) Plaque assay

The effect of a number of variables on plaque formation by AHSV on the different cell lines was determined. These included the composition and concentration of buffer used for viral adsorption, the time required for optimal plaque development, and the influence of the concentration of agarose used in the overlay. The addition to the overlay of serum at two different concentrations, of cortisol and of DEAE-dextran at various concentrations was also investigated in view of their reported stimulatory effect on plaque formation.



The final procedure that was adopted, based on the results of these investigations, is outlined below.

After rinsing the dishes with serum-free medium, cultures were inoculated with 0.1 ml of the appropriate virus dilution in 0.002M tris buffer. Adsorption of virus was allowed for 30 min at room temperature. Dilutions, which were different for the different cell lines, were selected to give about 60 plaques per dish. When viral infectivity titres were calculated, however, serial dilutions were employed and the titre based on the average number of plaques counted at the different dilutions. Experimental determinations were carried out in triplicate with one to three replicates per experiment. Results are expressed as numbers of plaque-forming units (PFU).

The normal overlay consisted of an autoclaved 2.4 per cent agarose suspension in Earle's saline which was diluted with Eagle's medium without serum to 0.24 per cent for the assay on BHK 21 cells and 0.40 per cent for the assay on L and VERO cells. The diluted suspensions were cooled to a temperature of 45°C before 4 ml of overlay were added per dish. Incubation was carried out at 37°C in a humidified atmosphere of 5 per cent CO<sub>2</sub> in air. Plaques were counted on BHK 21 cells after an incubation period of 3 days and on L and VERO cells after 5 to 6 days.

To facilitate the counting of the plaques BHK 21 cells were flooded with 0.3 per cent crystal violet in 0.9 per cent saline and those containing the L and VERO cells with 0.05 per cent neutral red in 0.5M phosphate buffer at pH 6.5. Bouin's fixative (Paul, 1965) was also satisfactory. Plaques were counted after 1 to 5h further incubation at 37°C.

(b) *End-point titration*

Roller-tube cultures were used as described by Oellermann *et al.* (1969) and the titre expressed as log TCID<sub>50</sub>/ml.

*Inhibition by Actinomycin D\**

Confluent monolayer cultures of all three cell lines in petri dishes were rinsed with Eagle's medium and the cultures pretreated for 60 min with 5 ml of medium containing the respective Actinomycin D concentration per dish. The medium was removed, the cultures inoculated with AHSV at 20 PFU/cell and again washed with fresh medium after an adsorption period of 1 h. Finally Eagle's medium containing the required concentration of Actinomycin D was added to the respective cultures, which were then incubated at 37°C. Infectivity titrations were carried out after 24 h when the control cultures showed advanced cytopathic effects on all three cell lines.

*Virus purification*

The method of virus purification used has been described previously (Oellermann *et al.*, 1969; Verwoerd, 1969).

*Isolation of double-stranded viral RNA*

The isolation procedures of double-stranded viral RNA from infected cells employed were described by Oellermann *et al.* (1969).

*Sucrose density gradient sedimentation analysis*

The procedure was similar to that described by Oellermann *et al.* (1969) except that sedimentation was carried out at 37,000 rpm (165,000 × g) for 16 h at 24°C in a Spinco SW 41 rotor.

*Polyacrylamide gel electrophoresis*

The electrophoretic procedure has been described in detail elsewhere (Oellermann *et al.*, 1969). Gels were prepared in Loening buffer (Loening, 1967) according to the method described by Verwoerd *et al.* (1970).

*Radioactive precursors and assay of radioactivity*

Labelling of viral RNA with <sup>3</sup>H-uridine (The Radio-chemical Centre, Amersham, England) at 1.0 μCi/ml was used throughout this investigation (Oellermann *et al.*, 1969). Assays of radioactivity were carried out as described by Verwoerd *et al.* (1970).

RESULTS

*Viral adsorption in different buffers*

The efficiency of plaque formation in tris buffer at different molarities was compared to that in PBS and 1/100 PBS. The results are presented in Table 1.

TABLE 1 *Influence of viral adsorption in different buffers on the efficiency of plaque formation*

Buffer	Time of adsorption (min)	Number of plaques		
		BHK21	L	VERO
0.002M tris	10	59	56	72
	30	65	58	75
0.02M tris	10	55	59	63
	30	54	65	59
0.2M tris	10	23	50	44
	30	35	52	51
1/100 PBS	10	16	21	18
	30	36	29	35
	120	15	16	7
PBS	10	5	19	5
	30	17	17	11
	120	19	14	23

It is interesting that an increase in the hypotonicity of the buffer resulted in a definite increase in the number of plaques formed. This applied to both tris and PBS. However, tris was superior to PBS at all concentrations tested. The most obvious difference between the buffers is one of pH, but this difference did not influence the efficiency of plaque formation (Oellermann, unpublished observation). Hopkins *et al.* (1967) observed a similar decrease in plaque formation on VERO cells with PBS compared to those with maintenance medium. No explanation for the observed difference between the buffers can be given.

When the adsorption time was extended to 2 h, degradation of the cell monolayer, with a concomitant decrease in the number of plaques formed, was observed in the hypotonic buffer. For routine purposes, therefore, adsorption was carried out in 0.002M tris for 30 min at room temperature.

*Rate of plaque formation under agarose*

The incubation time required for optimal plaque counts was determined. These results are illustrated in Fig. 1.

Occasionally maximum plaque numbers were found on BHK 21 cells after 2 days incubation at 37°C. On the average, however, an incubation period of 3 days was necessary for comfortable counting and maximum num-

\*Merck, Sharp and Dohme, New York

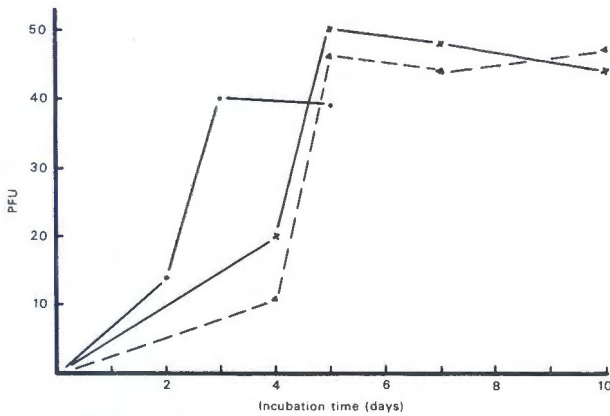


Fig. 1 Relationship between plaque formation and period of incubation for BHK21 cells • —•—, L cells ▲ —▲— and VERO cells x —x—.

ber of plaques. Incubation for longer periods resulted in a marked increase in plaque size, which interfered with accurate counting of plaques, and in a not infrequent degeneration of the cell monolayer.

In the case of L and VERO cells maximum counts were found after 5 days of incubation. Sometimes the plaques on L cells were still very small then but an increase in their numbers was never observed when the incubation time was increased to 7 or 10 days. The addition of a second agarose overlay to both L and VERO cells after 4 days resulted in larger plaques and an improved cell condition after incubation periods of 7 and 10 days, though no increase in plaque numbers was observed. After an incubation period of 10 days the increased plaque size resulted in their overlapping which made counts at this stage erratic.

For routine analysis therefore, plaques were counted on BHK 21 cells after 3 days and on L and VERO cells after 5 to 6 days at 37°C.

#### Influence of overlay on plaque formation

##### (a) Influence of agarose concentration

The effect of the concentration of the agarose overlay on plaque formation is illustrated in Fig. 2.

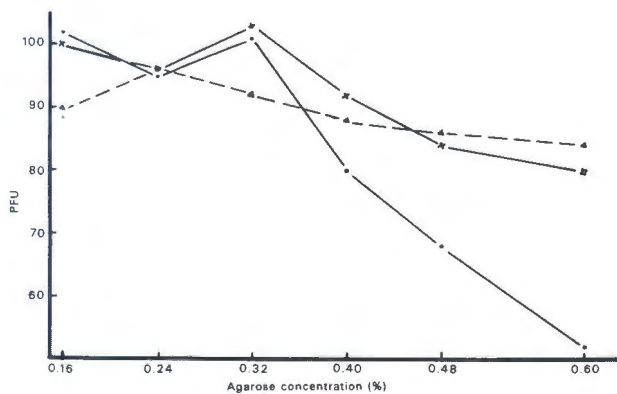


Fig. 2 Influence of agarose concentration on plaque formation of AHSV on BHK21 cells • —•—, L cells ▲ —▲— and VERO cells x —x—.

At agarose concentrations of 0.4 per cent and higher plaque formation on BHK 21 cells was markedly inhibited. This inhibition was particularly obvious with respect to plaque size and was more variable in its effect on plaque numbers. The inhibitory effect on L and VERO cells was only slight with respect to plaque num-

bers but plaque size decreased with an increase in agarose concentration. Similar results were reported for BTV on L cells (Howell, Verwoerd & Oellermann, 1967) and for AHSV on MS cells (Hopkins *et al.*, 1966).

In subsequent experiments 0.24 per cent agarose was used on BHK 21 cells whereas 0.4 per cent agarose was used on L and VERO cells.

##### (b) The influence of composition of overlay

A number of substances which stimulate plaque formation in some viruses was tested on AHSV. The results are summarized in Table 2.

TABLE 2 Influence of different supplements to the overlay on plaque formation

Overlay	Number of plaques		
	BHK 21	L	VERO
Normal (without serum) . . . . .	66	58	68
Normal + 0.5% serum . . . . .	58	55	76
Normal + 5.0% serum . . . . .	5	68	72
Normal + 0.25 µg Cortisol/ml . . . . .	64	40 × 10	64
Normal + 4 µg DEAE-dextran/ml . . . . .	56	53	72
Normal + 12 µg DEAE-dextran/ml . . . . .	54	52	70
Normal + 24 µg DEAE-dextran/ml . . . . .	52	48	60

The addition of serum to the medium up to a level of 5 per cent had no effect on plaque formation on L and VERO cells. However, serum at 5 per cent resulted in a ten-fold decrease in plaque numbers on BHK 21 cells. The low level of 0.5 per cent serum stimulated the metabolic activity of the cells which frequently resulted in excessive acidity of the petri dish cultures. In the presence of serum the overall plaque size was reduced and the supplementation with serum was not routinely employed in the plaque assay.

Cortisol at a concentration of 0.25 µg/ml stimulated plaque formation on L cells resulting in a five- to ten-fold increase in plaque numbers. Although it might merit inclusion in the overlay for L cells, the plaques formed were mostly fairly indistinct, which largely cancelled out any advantage in its use. Cortisol was therefore not normally included in the overlay medium for plaque assay.

The supplementation with DEAE-dextran at different concentrations did not improve the efficiency of plaque formation. Only occasionally was an increase in plaque size observed at concentrations of up to 12 µg/ml. This slight beneficial effect did not warrant the inclusion of DEAE-dextran in the overlay for the routine plaque assay.

##### Linearity

The results of the relationship between PFU and virus dilution are illustrated in Fig. 3.

From these data it is apparent that a good linear relationship exists between plaque numbers and relative virus concentration on all three cell lines employed. Similar observations were made by Mirchamsy & Taslimi (1966a) on MS cells. This evidence establishes the validity of this method of assay for single infective particle counts.

##### Plaque assay vs end-point titration

During the course of this work it was observed that the three cell lines differed in their susceptibility to infection by AHSV. This was further investigated by the simultaneous titration of AHSV at different stages of purity on the cell lines according to the two methods of assay described. The plaque morphology of AHSV on



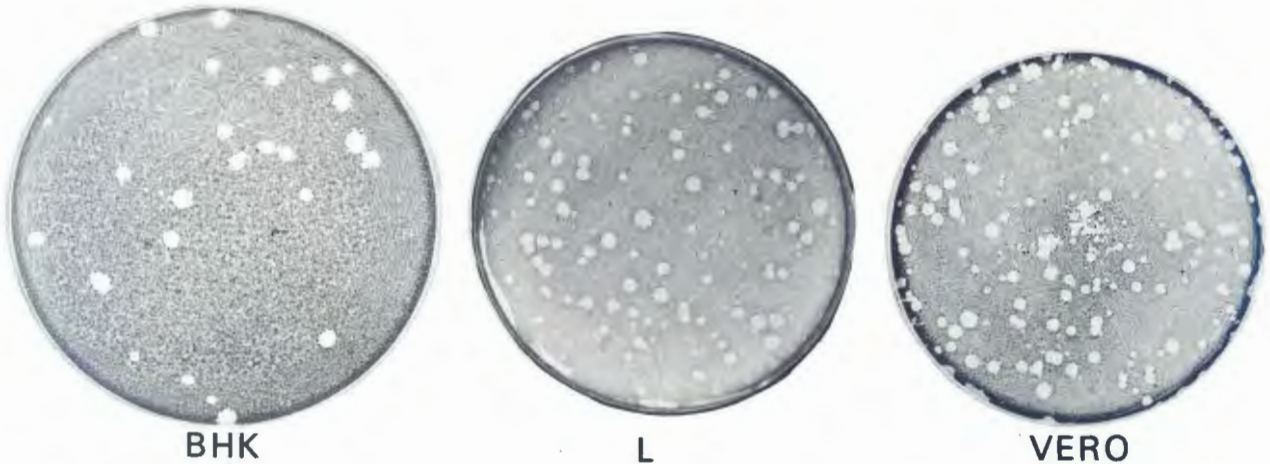


PLATE 1 Morphology of plaques produced by AHSV on BHK21, L and VERO cells. For photography the cell monolayers were superficially fixed with Bouin's fluid before removing the agarose overlay and rinsing the cells with water. BHK21 cells were stained with crystal violet and L and VERO cells with neutral red.

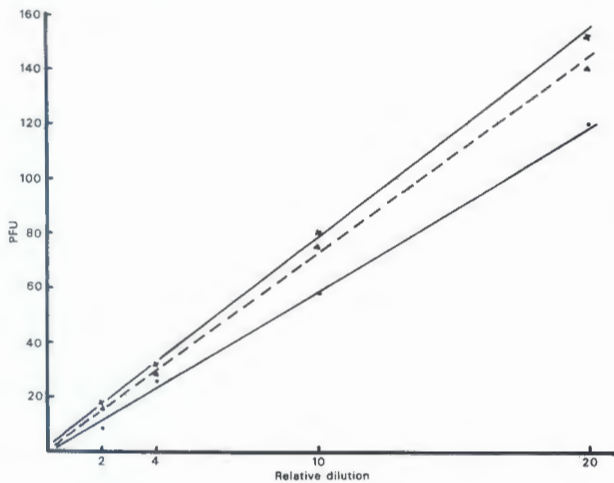


FIG. 3 Relationship between AHSV concentration and plaque formation on BHK21 cells • —••, L cells ▲ - - - ▲ and VERO cells x — x.

the respective cell lines is illustrated in Plate 1 and the virus titres are presented in Table 3.

No significant difference in the morphology of plaques on the different cell lines was observed but the variation in plaque size always occurred. The plaque-size variants do not seem to be true phenotypic variants although small-plaque isolates did tend to produce smaller plaques than were observed in the large-plaque isolates or "wild type" strain 9 AHSV (B. J. Erasmus, Veterinary Research Institute, Onderstepoort, personal communication).

The infective virus titres calculated from the end-point titration assay were similar to those based on the plaque assay for all three cell lines used. Large differences in infective virus titres can be detected by the method of end-point titration but the method of assay is not sensitive enough to give an accurate measure of smaller differences encountered in the inhibition of virus production by Actinomycin D.

Marked differences in the susceptibility to infection existed between the different cell lines. The titre on VERO cells was 4 to 6 times less and that on L cells 10 to 15 times less than that observed on BHK 21 cells. In the experiment on the inhibition of AHSV production by Actinomycin D a similar susceptibility gradient was observed which was practically independent of the cell line used for virus production. This difference in susceptibility to infection emphasizes the central role of the cell in viral infection.

*Inhibition by Actinomycin D*

Infective virus production on the different cell lines, in the presence of varying concentrations of actinomycin D, was determined by plaque assay on all three cell lines. The assay data on the three cell lines were identical and only the results on BHK 21 cells are illustrated in Fig. 4.

An increase in Actinomycin D concentration resulted in an increased inhibition of infective virus production. The inhibitory effect was practically identical on all three cell lines resulting in an inhibition of 80 to 90 per cent at an Actinomycin D concentration of 1.0 µg/ml. Although this inhibition is less than that reported previously (Oellermann *et al.*, 1969), the results are similar

TABLE 3 Comparison between plaque assay and end-point titration for infective virus on different cell lines

Stage of purification of virus assayed	Virus titre					
	BHK 21		L		VERO	
	PFU/ml	log TCID <sub>50</sub> /ml	PFU/ml	log TCID <sub>50</sub> /ml	PFU/ml	log TCID <sub>50</sub> /ml
Unpurified virus stock	1.0 × 10 <sup>8</sup>	7.5	8.0 × 10 <sup>6</sup>	6.5	2.1 × 10 <sup>7</sup>	7.5
Resuspended cell debris	1.1 × 10 <sup>9</sup>	8.25	9.5 × 10 <sup>7</sup>	7.5	3.0 × 10 <sup>8</sup>	8.0
Combined hypotonic buffer extract	4.5 × 10 <sup>8</sup>	8.25	4.1 × 10 <sup>7</sup>	7.5	8.0 × 10 <sup>7</sup>	7.5
After fluorocarbon treatment	3.0 × 10 <sup>8</sup>	8.0	2.0 × 10 <sup>7</sup>	7.0	6.0 × 10 <sup>7</sup>	7.5
After Tween 80-ether treatment	2.0 × 10 <sup>8</sup>	8.0	2.0 × 10 <sup>7</sup>	7.25	5.0 × 10 <sup>7</sup>	7.5
Purified viral pellet	7.5 × 10 <sup>9</sup>	9.0	6.0 × 10 <sup>8</sup>	8.25	1.2 × 10 <sup>9</sup>	8.5

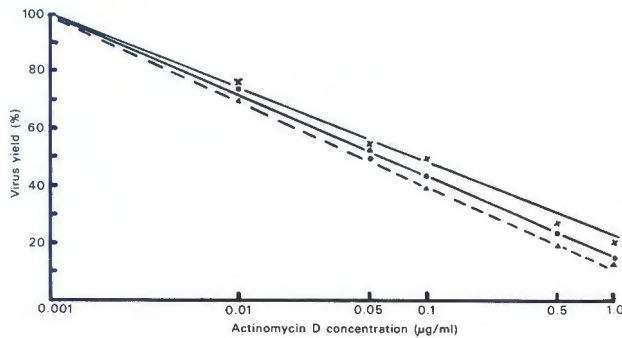


FIG. 4 Influence of different concentrations of Actinomycin D on infective AHSV production on BHK21 cells • —••, L cells ▲ — — — ▲ and VERO cells x — — — x.

to the inhibition of BTV on BHK 21 cells reported by Verwoerd (1969). This supports the double stranded nature of the RNA genome of AHSV.

#### Analysis of viral RNA

##### (a) Sucrose gradient sedimentation analysis

In an earlier investigation (Oellermann *et al.*, 1969) the presence of five poorly resolved components was indicated. In an attempt to improve the resolution, centrifugation of the  $^3\text{H}$ -uridine labelled viral RNA was carried out on a 15 to 30 per cent sodium dodecyl sulphate (SDS)-containing sucrose gradient as described under methods. The absorbance pattern at  $260\text{ m}\mu$  (Fig. 5a) shows five definite components at the position of 8.5, 10, 12, 13.5 and 14.5S. For further reference the position of 15.5S is also indicated. The corresponding distribution of radioactivity is illustrated in Fig 5b. Included in this figure is the radioactive distribution pattern of a ribonuclease (RNase)-treated RNA sample. From both curves it is evident that at least six RNase-resistant components exist with sedimentation constants corresponding to 8.5, 10, 12, 13.5, 14.5 and 15.5S.

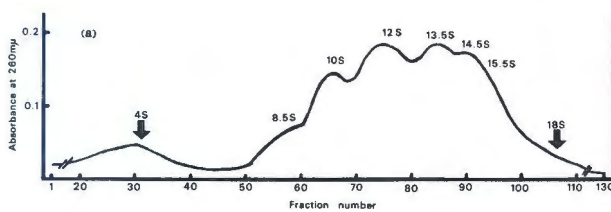


FIG. 5 (a) Absorbance at  $260\text{ m}\mu$  of a sucrose gradient after sedimentation of AHSV-RNA.

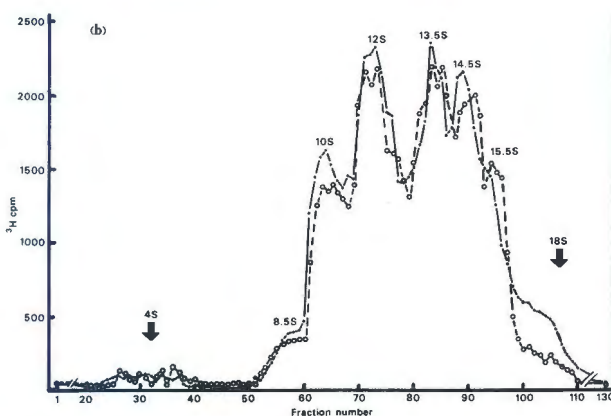


FIG. 5 (b) Distribution of radioactivity in a sucrose gradient sedimentation analysis of AHSV-RNA before • —•• and after o — — — o hydrolysis with RNase. Labelled BHK21 cell RNA markers were used.

The molecular weights of the individual components were calculated on the basis of the relationship  $S = 0.0882 \times (\text{molecular weight})^{0.346}$  deduced by Studier (1965) for double-stranded DNA. The data are presented in Table 4.

##### (b) Polyacrylamide gel electrophoresis

In an earlier investigation on AHSV, the RNA genome was resolved into six components. In the present investigation an improved resolution was achieved by employing Loening buffer for making the gel as well as using it as reservoir buffer. The gels were cut into 1 mm fractions and the distribution of radioactivity for both RNase-treated and -untreated RNA is presented in Fig. 6.

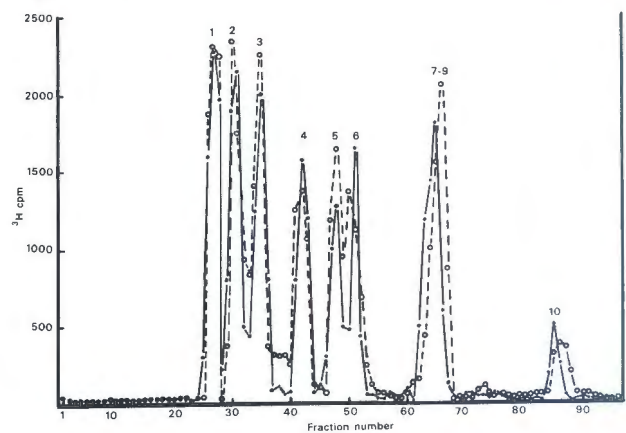


FIG. 6 Separation of AHSV-RNA by polyacrylamide gel electrophoresis before • —•• and after o — — — o treatment with  $2\text{ }\mu\text{g}$  RNase/ml at  $37^\circ\text{C}$  for 30 min.

The data revealed eight RNase-resistant components numbered 1 to 10. Fractionation on 3.6 per cent gels did not improve the resolution. However, under identical conditions, BTV-RNA was resolved into 10 components (Verwoerd *et al.*, 1970).

The molecular weights of the double-stranded RNA components of reovirus have been determined (Bellamy *et al.*, 1967; Shatkin *et al.*, 1968; Watanabe & Graham, 1967). The average molecular weights of the major components resolved by polyacrylamide gel electrophoresis were plotted against the distance they migrated into the gel. A linear relationship was obtained (Verwoerd *et al.*, 1970) which was used as reference curve to estimate the molecular weights of the AHSV-RNA segments. The values obtained for gel peaks 1 to 10 (Fig. 6) are presented in Column 3 of Table 4.

If a random incorporation of radioactive label into the entire viral genome occurs, the counts per min per RNA segment should be proportional to the molecular weight of the segment. An analysis of the radioactivity distribution among the peaks in Fig. 6 (AHSV-RNA not treated with RNase) was therefore carried out. The total counts per min for all peaks were divided by the total estimated molecular weight of  $1.5 \times 10^7$  and the resultant figure multiplied by the estimated molecular weight of each peak to obtain the estimated counts per min per peak. These values are given in Table 4 (Columns 4 and 5). There is good agreement between the estimated and observed counts except for gel peak numbered 7 to 9. For this peak the estimated counts per min corresponding to a molecular weight of  $0.88 \times 10^6$  were 1873. However, 5658 counts per min were observed which is equivalent to three times the estimated counts. It is therefore reasonable to assume that three



TABLE 4 *Molecular weight of AHSV-RNA components estimated from polyacrylamide-gel-electrophoretic mobility and compared with values calculated from density gradient sedimentation analysis*

Gel peak No.	Distance migrated (mm)	Estimated molecular weight (daltons)	Estimated counts/min	Observed counts/min	Sedimentation constant (svedbergs)	Calculated molecular weight (daltons)
1.	27	$2.8 \times 10^6$	5960	6150	15.5	$3.0 \times 10^6$
2.	30.5	$2.5 \times 10^6$	5322	5350	14.5	$2.5 \times 10^6$
3.	35	$2.1 \times 10^6$	4471	4500	13.5	$2.1 \times 10^6$
4.	42	$1.7 \times 10^6$	3619	3575	12	$1.5 \times 10^6$
5.	47	$1.5 \times 10^6$	3193	3200		
6.	51	$1.3 \times 10^6$	2768	2565	10	$0.86 \times 10^6$
7-9.	64	$0.88 \times 10^6$	1873	5658		
10.	85	$0.46 \times 10^6$	979	935		
				31,933		

RNA segments with an average molecular weight of  $0.88 \times 10^6$  per segment must be present in the AHSV genome. This value is similar to the average of peaks 7 to 9 of BTV-RNA with molecular weights of 0.90, 0.80 and  $0.74 \times 10^6$  respectively.

The molecular weights of the remaining RNA segments of AHSV and BTV as determined by Verwoerd *et al.* (1970) were found to be remarkably similar.

The molecular weights calculated from the sedimentation constants on sucrose gradients (Fig. 5) agree well with the estimated values based on the electrophoretic mobility. The 12S component with a calculated molecular weight of  $1.5 \times 10^6$  corresponds to the average estimated for gel peaks 4 to 6. These data therefore suggest that the molecular weights of the RNA components of AHSV are, within limits, accurate estimations.

DISCUSSION

The end-point titration of AHSV is carried out by microscopic examination of roller-tube cultures of BHK21 cells after 4 to 5 days and that of L and VERO cells after 7 to 10 days. From data presented in this paper it is also evident that this assay procedure is not sufficiently accurate to detect small differences in infectivity titres of virus. Such small differences, as were encountered in the inhibition of virus production by Actinomycin D, can be determined by means of the plaque assay. Results from the plaque assay of AHSV on BHK21 cells are available after 3 days and on L and VERO cells after 5 to 6 days. The advantage of the plaque assay over the end-point titration is therefore obvious and does not require further elaboration.

A considerable difference was observed in the behaviour of the different cell lines to infection by AHSV. Highest infectivity titres were always found in the assay on BHK21 cells. The assay on VERO cells yielded titres intermediate to those on BHK21 and L cells. These differences were practically independent both of the cell line on which the virus was produced and of the purity of the virus. It would therefore appear as if comparative numbers of complete virus particles were assembled per cell and that the difference between the cell lines in this respect is relatively small. This emphasizes the significance of the cell membrane in viral infection.

The morphological characteristics of AHSV and BTV are very similar (Els & Verwoerd, 1969; Oellermann *et al.*, 1969). However, no serological cross-reaction occurs and according to Verwoerd & Huisman (1969) only a small amount of hybridization occurs between these two viruses. Bluetongue virus produced on BHK21 and assayed on BHK21 and L cells, gave

similar titres on both cell lines (Oellermann, unpublished observation) and the difference in cell susceptibility to viral infection was not observed. It is therefore apparent that there is a significant difference between these two viruses in the surface specificity of the viral coat protein.

The improved resolution of the RNA segments in sucrose gradient sedimentation analysis reported in this study revealed at least six RNase-resistant peaks with sedimentation constants of 8.5, 10, 12, 13.5, 14.5 and 15.5S. By means of polyacrylamide gel electrophoresis the RNA was resolved into eight components. By comparison of the molecular weights calculated from the sedimentation constants and the values estimated from their electrophoretic mobility it is obvious that Gel Component 1 corresponds to the 15.5S peak, Component 2 to 14.5S, Component 3 to 13.5S, Components 4, 5 and 6 to 12S, Component numbered 7 to 9 to peak 10S and Component 10 to peak 8.5S. From the radioactivity data it was determined that three segments with an average molecular weight of  $0.88 \times 10^6$  daltons per segment are present in the AHSV genome. This constitutes a major detectable difference from BTV, which has three clearly resolved components in this size group with an average sedimentation constant of 10 svedbergs. Comparative data between BTV and reovirus have been discussed in another paper (Verwoerd *et al.*, 1970). A very close relationship with respect to the size distribution of the RNA segments has been observed between AHSV and BTV, although they are responsible for marked functional differences.

Although significant differences in the viral structure and specificity of the protein coat exists between AHSV, BTV and reovirus, experimental evidence supports a remarkable similarity between the segmented, double-stranded RNA genome of these viruses (Oellermann *et al.*, 1969; Shatkin *et al.*, 1968; Verwoerd *et al.*, 1970). The total molecular weight of  $1.5 \times 10^7$  daltons estimated per viral genome is the same for all three viruses. Segmentation into 10 pieces is also similar in spite of differences with respect to their size. It is at this stage not possible to assess fully the significance of the interesting properties of this group of viruses and a detailed investigation into the functional aspects of the double-stranded RNA genome is necessary.

SUMMARY

Experimental evidence in support of a plaque assay for AHSV was presented. Three cell lines: BHK21, L and VERO, were used of which BHK21 was the most satisfactory. The assay procedure was compared with the method of end-point titration and the superiority of the plaque assay demonstrated.

It was shown that increased Actinomycin D concentrations resulted in increased inhibition of infective virus production. The inhibitory effect was very similar on all three cell lines resulting in an 80 to 90 per cent inhibition at an Actinomycin D concentration of 1.0  $\mu\text{g/ml}$ .

By sucrose gradient sedimentation analysis it was possible to resolve AHSV-RNA into six peaks with sedimentation constants of 8.5, 10, 12, 13.5, 14.5 and 15.5S. Eight components were resolved by polyacrylamide gel electrophoresis. The molecular weights of the components estimated from polyacrylamide-gel-electrophoretic mobilities agreed well with the values calculated from the sedimentation constants. The molecular weight of the largest component was estimated at  $2.8 \times 10^6$  and that of the smallest at  $0.46 \times 10^6$  daltons. It was shown that three segments almost identical in size and with an average molecular weight of  $0.88 \times 10^6$  per segment were present resulting in 10 segments per AHSV genome.

Attention was focussed on the significant similarities and differences observed between AHSV, BTV and reovirus.

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