Onderstepoort J. vet. Res. 43 (4), 193–200 (1976)

A GEL ELECTROPHORETIC STUDY OF THE PROTEIN AND NUCLEIC ACID COMPONENTS OF AFRICAN HORSESICKNESS VIRUS

CHRISTINA W. BREMER, Veterinary Research Institute, Onderstepoort

ABSTRACT

BREMER, CHRISTINA W., 1976. A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. *Onderstepoort Journal of Veterinary Research* 43 (4), 193–200 (1976).

The physico-chemical structure of African horsesickness virus (AHSV) is compared with that of some of the other members of the Reoviridae, and in particular with that of bluetongue virus (BTV), the type strain of the orbivirus genus. This study adduces evidence of a great similarity between the gel electrophoretic patterns of the polypeptides of AHSV and BTV. The molecular mass values of the 7 AHSV polypeptides range between $0,30 \times 10^5$ and $1,46 \times 10^5$ dalton, a variation similar to that of BTV. The close relation between AHSV and BTV is further affirmed by the gel electrophoretic resolution of the AHSV double-stranded RNA genome into 10 segments.

Résumé

UNE ÉTUDE EN ÉLECTROPHORÈSE SUR GEL DES COMPOSANTS PROTÉIQUES ET EN ACIDES NUCLÉIQUES DU VIRUS DE LA PESTE ÉQUINE

L'auteur a comparé la structure physico-chemique du virus de la peste équine (VPE) avec celle des autres virus des Reoviridae et plus particulièrement avec celle du virus de la peste ovine (VPO), la souche de type du genre des orbivirus. Cette étude ajoute preuve de la grande similitude des modèles des polypeptides du VPE et du VPO en électrophorèse sur gel. Le poids moléculaire des 7 polypeptides du VPE se situe entre $0,30 \times 10^5$ *et* $1,44 \times 10^5$ *dalton, une variation correspondante avec celle du VPO. La résolution en électrophorèse sur gel du génome de l'ARN double du VPE en* 10 *segments affirme le rapport étroit entre le VPE et le VPO.*

INTRODUCTION

Electron miscroscopic examination of African horsesickness virus (AHSV), a member of the orbivirus genus of the Reoviridae (Melnick, 1975), establishes its great similarity to bluetongue virus (Els & Verwoerd, 1969; Oellermann, Els & Erasmus, 1970), in that they both contain a segmented double-stranded (ds) RNA genome. Previous studies, utilizing polyacrylamide gel electrophoresis, indicate that AHSV contains 10 ds RNA genome segments although only 8 of these could be resolved (Oellermann, 1970). In the present investigation attempts were made to obtain a better resolution of the RNA components, and the protein components were studied for the first time. Molecular mass determinations of the different components yielded information about the coding relation between AHSV RNA segments and the protein components.

MATERIALS AND METHODS

Cells and media

BHK-21 and mouse fibroblast cells were used (Verwoerd, Oellermann, Broekman & Weiss, 1967). Cells were grown in BHK-21 medium (MacPherson & Stoker, 1962).

Virus

AHSV type 3 (strain 13/63), isolated as described by Howell (1962), was passaged 60 times intracerebrally in suckling mice, cloned 3 times by plaque isolation in monolayer cells, freeze-dried and stored until it was used as an inoculum. BTV type 10 A (Howell, 1969) and reovirus type 3 were used.

Virus assay

Two different plaque tests were used for the assay of AHSV (Oellermann, 1970) and BTV (Howell, Verwoerd & Oellermann, 1967).

Virus production and purification

For virus production BHK-21 monolayer cells were infected at a multiplicity of approximately 5–10 PFU per cell and harvested after 36–48 h.

Received 24 August 1976—Editor

The method used for this purification of AHSV, BTV and reovirus, followed that previously described by Verwoerd, Els, De Villiers & Huismans (1972). The steps included Freon extractions and an ether extraction followed by banding of virus on sucrose gradients. Only where specified was the virus further purified on cesiumchloride (CsCl) density gradients.

Labelling of RNA with ^{3}H-adenine

Cell monolayers in roller bottles were infected with approximately 30 plaque-forming units (PFU) of reovirus or AHSV per cell. After 3 h the growth medium in each bottle was replaced by serum-free BHK-21 medium containing 1 μCi/ml of ^{3}H-adenine. Virus was harvested after 24 h, purified and, after dissociation, electrophoresed in a cylindrical polyacrylamide gel.

Isolation of viral double-stranded RNA from infected cells

Cells were harvested 48 h after infection with the virus. A cytoplasmic extract was prepared according to the method of Bellamy, Shapiro, August & Joklik (1967). Sodium dodecyl sulphate (SDS) was added to the cytoplasmic extract to give a final concentration of 0,5%, and sodium acetate (NaAc) and ethylenediamine-tetra-acetic acid (EDTA) were both added to give final concentrations of 0,01 M. The pH of the solution was adjusted to 5,0 with acetic acid and this was followed by two phenol extractions, after the method of Scherrer & Darnell (1962). Phenol residues were removed with an ether extraction and the RNA precipitated by the addition of 2 volumes of ethanol and sodium chloride to a final concentration of 0,1 M.

The precipitate was dissolved in 0,01 M STE buffer [0,01 M Tris (hydroxy-methyl) aminomethane, 0,001 M EDTA and 0,01 M NaCl] and single-stranded RNA was removed from the solution by salt precipitation at 1,25 M LiCl. The ds RNA was then further purified on a methylated albumin kieselguhr column (Verwoerd & Huismans, 1969).

Polyacrylamide gel electrophoresis

Protein gel electrophoresis: A vertical slab gel electrophoresis unit (Studier, 1973) was used. Polyacrylamide gels consisted of 7,5% polyacrylamide,

0,2% bisacrylamide, 0,1% SDS, 0,02 M EDTA, 0,1 M sodium phosphate buffer (pH 7,3), 0,1% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0,08% ammonium peroxydisulphate in 5,83 M urea. The electrode buffer consisted of 0,1% SDS, 0,02 M EDTA and 0,1 M sodium phosphate buffer (pH 7,3). Samples of 20–100 μg virus in 5 μl 2 mM Tris buffer were prepared for electrophoresis by the addition of 20 μl of a solution containing 4% 2-mercaptoethanol (2-ME) and 1,6% SDS in 8 M urea.

The mixture was kept at 70 °C for 1 min and then placed in the sample slots. Electrophoresis was carried out for 22 h at 3 V/cm, 30 mA and 1 000 pulses per min. An Ortec 4 100 pulsed constant power supply was used. Gels were stained with Coomassie Brilliant Blue (CBB) G-250 (Anderson, Cawston & Cheeseman, 1974).

RNA gel electrophoresis: Electrophoresis of RNA on both slab and cylindrical gels was based on the method of Loening (1967). The slab gels consisted of 7% polyacrylamide, 0,186% bisacrylamide, 0,1% TEMED and 0,08% ammoniumperoxydisulphate in Loening buffer (0,04 M Tris, 0,02 M NaAc and 2 mM EDTA, pH 7,8). A drop of glycerol was added to 5–10 μg of ds RNA in 10 μl 0,01 M STE buffer, and the sample was then placed in the sample slot. Loening buffer was used in the reservoir. Electrophoresis was carried out for 28 h at 4,5 V/cm, 20 mA and 1 000 pulses per min. Gels were fixed in 1 M acetic acid, stained in 0,4% methylene blue in 0,2 M NaAc pH 4,7 and destained in water.

Electrophoresis of labelled AHSV RNA was carried out by using cylindrical gels 20 cm long containing 3% polyacrylamide. Electrophoresis continued for 5 h at 100 V 10 mA/gel. Gels were sliced into 1 mm pieces, each piece being placed in 0,2 ml of 10% pipiridine solution and each sample counted for 10 min in Kinard's scintillator solution (Kinard, 1957), in a Packard Tri-Carb Scintillation spectrometer.

Scanning of electrophoretograms

Stained gels were scanned with a Vitatron Model TLD flying spot densitometer.

Molecular mass determinations

Molecular mass values of polypeptides were determined as described by Dunker & Rueckert (1969), Shapiro, Viñuela & Maizel (1967) and Weber & Osborn (1969). Bluetongue virus polypeptides (Verwoerd *et al.*, 1972) were used as standards. The molecular mass values of the AHSV ds RNA segments were determined by using BTV ds RNA (Verwoerd *et al.*, 1972) as standards.

Results

Electrophoretic separation of AHSV polypeptides

Purified AHSV and BTV, obtained from sucrose gradient bands, were dissociated and electrophoresed, as described under "Materials and Methods". A stained gel is shown in Fig. 1. The polypeptide pattern and the distribution of major and minor components show a remarkable similarity to that of BTV. The polypeptides were numbered from 1–7, in the order of decreasing molecular mass values. A distinction can be made between major polypeptides 2, 3, 5 and 7, which are present in relatively large amounts, and minor polypeptides 1, 4 and 6, which are present in smaller quantities. Only bands 2, 3 and 6 differ markedly from the corresponding BTV bands. A band with molecular mass slightly less than that of polypeptide 4 was often found. The fact that the total amount present of both this band and band 4 seemed to remain more or less constant in different samples, suggests that the smaller molecular mass band could possibly be a breakdown product of polypeptide 4. Alternatively, the presence of defective interfering particles with a slightly different protein constitution could explain the phenomenon.

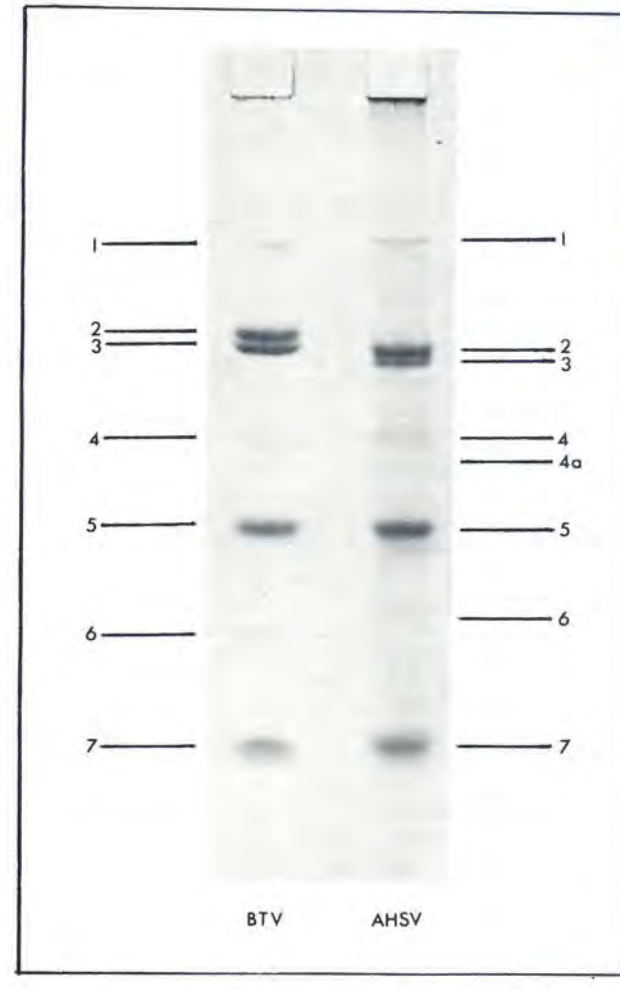

FIG. 1 A comparison of the polypeptide patterns of purified AHSV and BTV obtained by electrophoresis on 7,5% polyacrylamide gels. Gels were stained in a CBB G-250 solution and photographed.

CsCl density gradient centrifugation of AHSV

The BTV particle consists of an inner nucleocapsid surrounded by a diffuse outer protein layer of polypeptides 2 and 5 (Verwoerd *et al.*, 1972) which can be removed by CsCl density gradient centrifugation at pH 7. To determine if the outer polypeptides of AHSV could be removed by a similar method, samples of AHSV were centrifuged through CsCl gradients at 2 different pH values. The resulting virus bands were then analysed by gel electrophoresis (Fig. 2). Virus samples which banded at density $1,37 \pm 0,01$ on pH 8 gradients yielded gel electrophoretic patterns which indicated the presence of the normal 7 polypeptides, whereas the pattern obtained from virus which banded at $1,40 \pm 0,01$ on pH 6 gradients, showed the complete loss of polypeptide 3 and most of polypeptide 5. In some experiments virus banded on pH 8 gradients showed some loss of polypeptide 3, with no apparent reduction of polypeptide 5.

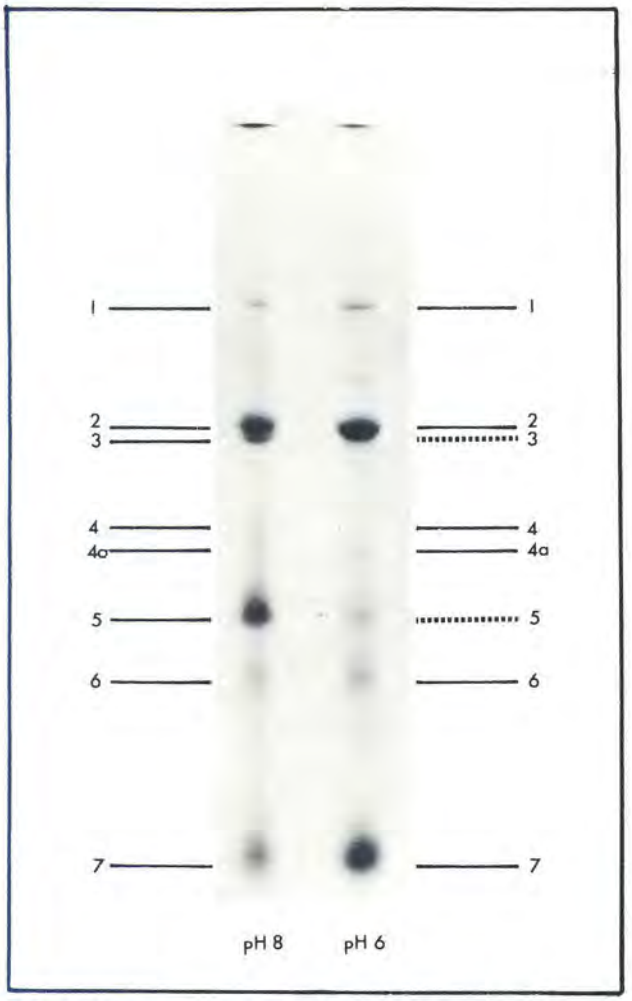

FIG. 2 The polyacrylamide gel electrophoretic patterns of purified AHSV obtained after density gradient centrifugation on CsCl gradients at pH 8 and pH 6

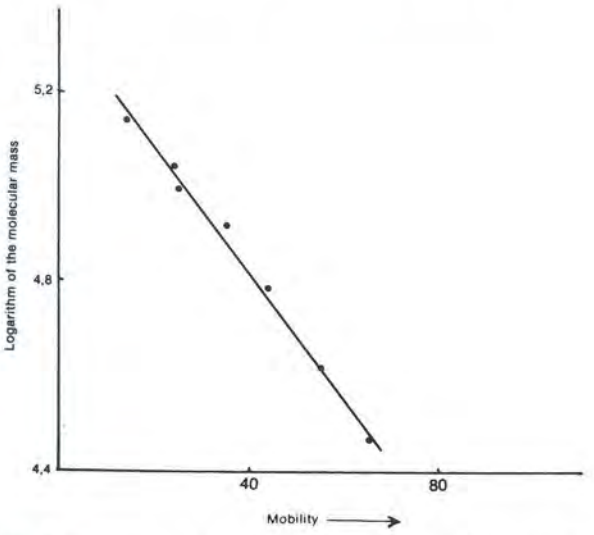

FIG. 3 The relation between the logarithm of the molecular mass and the mobilities of BTV polypeptides electrophoresed on 7,5% polyacrylamide gels

Molecular mass determination of the AHSV polypeptides

Within certain limits a linear relation exists between the logarithm of the molecular mass of polypeptides and their respective mobilities on SDS-containing acrylamide gels (Dunker & Rueckert, 1969; Shapiro *et al.*, 1967; Weber & Osborn, 1969). BTV and AHSV polypeptides were co-electrophoresed on such a polyacrylamide gel. The migration of BTV polypeptides was used to obtain a standard plot of the logarithm of molecular mass v. mobility (Fig. 3). From this plot the molecular mass values of AHSV polypeptides, summarized in Table 1, were determined.

TABLE 1 Theoretical and experimental molecular mass values of the polypeptides of African horsesickness virus (AHSV)

Molecular mass values BTV polypeptides*	Molecular mass values AHSV polypeptides		Equivalent genome segments
	Found	Calculated	
1. 140 000.....	1. 146 000	1. 141 000	1
2. 110 000.....	2. 103 000	2. 110 000	2
3. 101 000.....	3. 100 000	3. 92 000	3
4. 82 000.....	4. 78 000	4. 73 000	4
	4a 73 000		
5. 61 000.....	5. 59 000	5. 61 000	5
6. 42 000.....	6. 45 000	6. 57 000	6
		7. 34 000	7
7. 29 500.....	7. 30 000	8. 33 000	8
		9. 31 000	9
		10. 14 000	10

*Verwoerd *et al.*, 1972

Electrophoretic characterization of the double stranded RNA of AHSV

AHSV ds RNA was extracted from infected cells and characterized by polyacrylamide gel electrophoresis. The presence of 10 different segments could be demonstrated, when stained with a methylene blue solution (Fig. 4). The genome segments were numbered from 1–10 in the order of decreasing molecular mass values. It can be seen that segments 7, 8 and 9 are grouped very closely together.

In addition to the 10 ds RNA segments, reovirus also contains a fraction consisting of low molecular mass, single-stranded oligonucleotides (Bellamy *et al.*, 1967; Shatkin & Sipe, 1968) some of which contain only adenylate bases. It was of importance to determine if AHSV RNA resembled reovirus in this respect. ^{3}H-adenine labelled RNA from reovirus and AHSV were released from the respective viruses by dissociation in the presence of SDS, 2-ME and urea and electrophoresed on separate cylindrical gels. Gels were fractionated and the radioactivity in each fraction was determined (Fig. 5). A band of labelled RNA material, which electrophoresed faster than the smallest ds RNA segment, was found in the case of reovirus. No such component was present in AHSV RNA.

Molecular mass determination of AHSV RNA segments

The electrophoretic separation of RNA segments on polyacrylamide gels was used to determine the molecular mass of the different components of AHSV RNA. The revised values for the BTV RNA components, as obtained by Verwoerd *et al.* (1972), were used as standards. Electrophoresis of purified AHSV and BTV RNA was carried out as described. A stained gel is shown in Fig. 4. The gels were scanned, the migrational distances of each component measured and the linear relation between the mobilities of BTV RNA segments and the logarithm of their respective molecular mass values plotted (Fig. 6).

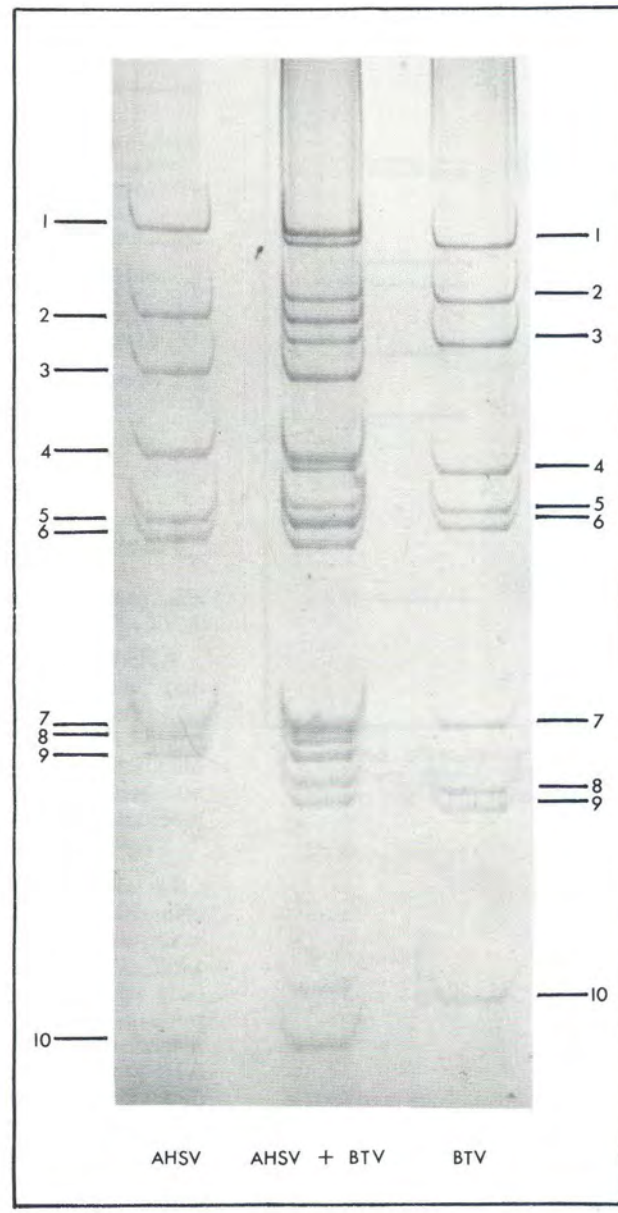

FIG. 4 A comparison of the ds RNA patterns of AHSV and BTV obtained by electrophoresis on 7% polyacrylamide gels. Gels were stained with methylene blue and photographed

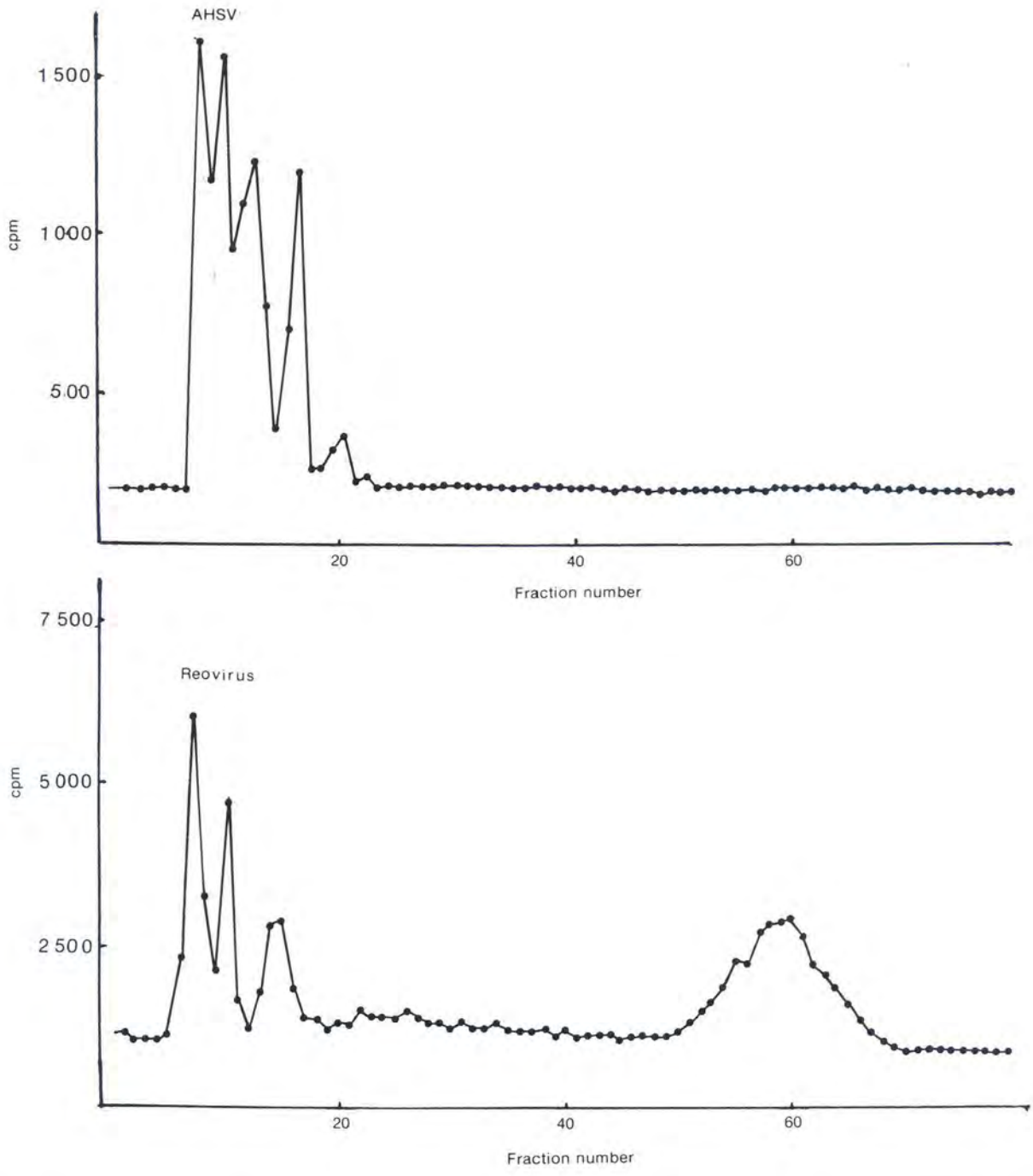

FIG. 5 Gel electrophoretic separation of low molecular mass RNA components of AHSV and reovirus labelled with ^{3}H-adenine

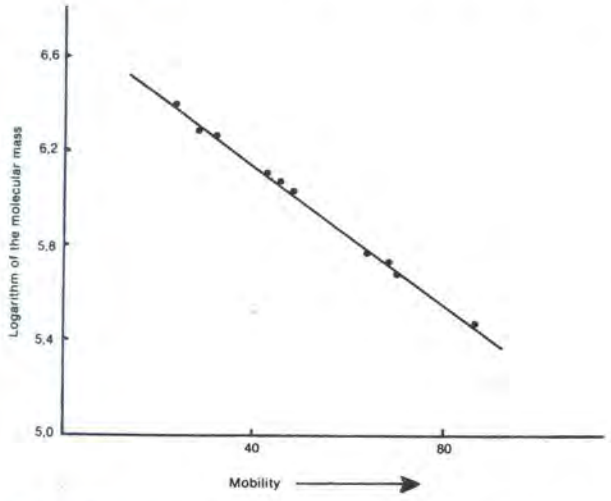

FIG. 6 The relation between the logarithm of the molecular mass values of the double-stranded RNA segments of BTV and their mobilities on 7% polyacrylamide gels

From this plot the molecular mass values of the AHSV RNA components were determined. When very small amounts of AHSV and BTV were co-electrophoresed in the same gel slot, optimum resolution was obtained and 20 different stained bands could be distinguished. No single AHSV RNA segment is therefore exactly identical with that of a corresponding BTV segment. The molecular mass values obtained are summarized in Table 2.

TABLE 2 Molecular mass values of the double-stranded RNA segments of African horsesickness virus (AHSV) and bluetongue virus (BTV)

Segment	AHSV-RNA	Segment	BTV-RNA
1	$2{,}53\times10^6$	1	$2{,}50\times10^6$
2	$1{,}98\times10^6$	2	$1{,}99\times10^6$
3	$1{,}66\times10^6$	3	$1{,}82\times10^6$
4	$1{,}32\times10^6$	4	$1{,}31\times10^6$
5	$1{,}10\times10^6$	5	$1{,}16\times10^6$
6	$1{,}03\times10^6$	6	$1{,}08\times10^6$
7	$0{,}61\times10^6$	7	$0{,}60\times10^6$
8	$0{,}59\times10^6$	8	$0{,}54\times10^6$
9	$0{,}57\times10^6$	9	$0{,}50\times10^6$
10	$0{,}24\times10^6$	10	$0{,}30\times10^6$

Discussion

In the orbivirus genus, only BTV was characterized in any great detail with regard to both the RNA and protein structure. It was found that, like BTV, AHSV RNA consisted of 10 double-stranded RNA segments. None of the AHSV segments was identical with a corresponding BTV RNA segment, however, and the AHSV pattern could very easily be distinguished from that of BTV, mainly because segments 7, 8 and 9 of AHSV electrophoresed characteristically close together. AHSV furthermore resembled BTV in that it contained no single-stranded adenine-rich oligonucleotide component, as was found in reovirus (Bellamy *et al.*, 1967; Shatkin & Sipe, 1968; Koide, Suzuka & Sekiguchi, 1968).

Even more so than in the case of the RNA, the electrophoretic pattern of the polypeptides of purified AHSV resembled that of BTV (Fig. 1). The relative amounts of the 4 major and 3 minor components were also very similar. The pattern differed significantly from that found for reovirus (Loh & Shatkin, 1968; Smith, Zweerink & Joklik, 1969).

The molecular mass values of the polypeptides which could be coded for by the AHSV genome segments were calculated according to Smith *et al.* (1969). A comparison of these theoretical values with those found experimentally is shown in Table 1. It can be assumed that the first 7 AHSV segments code for the 7 capsid polypeptides. RNA segments 8 or 9 can code equally well for polypeptide 7, however.

In the case of BTV, 2 polypeptides could be removed selectively by centrifugation of the virus through CsCl gradients at pH 7. It was shown by electron microscopic examination that these 2 polypeptides are part of the outer protein layer which covers the capsomere structure of the inner capsid particle (Verwoerd *et al.*, 1972). Preliminary electron microscopic examination of AHSV from infected cells revealed the presence of two types of particles (Bremer & Els, unpublished data, 1975). One of these was a particle which showed irregular surface structure, and the other a smaller nucleocapsid particle with clear capsomere structure. Results obtained from gel electrophoretic analysis of AHSV purified through CsCl gradients at pH 6 indicated the removal of polypeptides 3 and 5. On electron microscopic examination, these particles obtained from pH 6 gradients exhibited clear capsomere structure and resembled the nucleocapsid particles. From this information it can be concluded that AHSV has an inner capsid particle containing polypeptides 1, 2, 4, 6 and 7, surrounded by an outer protein layer consisting of polypeptides 3 and 5. In the case of BTV, the outer protein layer consisted of polypeptides 2 and 5. Polypeptides 2 of BTV and 3 of AHSV differed considerably in molecular mass. The most significant difference in polypeptide structure between AHSV and BTV was therefore found in the outer protein layer. The molecular mass values of the inner capsid polypeptides were remarkably similar. Polypeptides 2 of AHSV and 3 of BTV were almost identical. The only significant difference in molecular mass values of the respective inner capsid polypeptides seemed to be between polypeptides 6 of the two viruses.

Apart from these small physico-chemical differences, the 2 viruses appear to be very similar, further justifying their classification in the same genus.

Acknowledgements

The author wishes to thank Drr H. Huismans and D. W. Verwoerd and Prof. O. W. Prozesky for their guidance and support during this study, and Messrs P. A. M. Wege and J. G. van Gass for supplying the cell cultures.

This article is an extract from an M.Sc. thesis submitted to the University of Pretoria.

References

ANDERSON, M., CAWSTON, T. & CHEESEMAN, G. C., 1974. Molecular-weight estimates of milk-fat-globule-membrane protein-sodium sulphate complexes by electrophoresis in gradient acrylamide gels. *Biochemical Journal*, 139, 653–660.

BELLAMY, A. R., SHAPIRO, LUCILLE, AUGUST, J. T. & JOKLIK, W. K., 1967. Studies on reovirus RNA. I. Characterization of reovirus genome RNA. *Journal of Molecular Biology*, 29, 1–17.

DUNKER, A. K. & RUECKERT, R. R., 1969. Observations on molecular weight determinations on polyacrylamide gel. *Journal of Biological Chemistry*, 244, 5074–5080.

ELS, H. J. & VERWOERD, D. W., 1969. Morphology of bluetongue virus. *Virology*, 38, 213–219.

HOWELL, P. G., 1962. The isolation and identification of further antigenic types of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 29, 139–149.

HOWELL, P. G., 1969. The antigenic classification of strains of bluetongue virus, their significance and use in prophylactic immunization. D.V.Sc. Thesis, University of Pretoria.

HOWELL, P. G., VERWOERD, D. W. & OELLERMANN, R. A., 1967. Plaque formation by bluetongue virus. *Onderstepoort Journal of Veterinary Research*, 34, 317–332.

KINARD, F. E., 1957. Liquid scintillator for the analysis of tritium in water. *Review of Scientific Instruments*, 28, 293–297.

KOIDE, F., SUZUKA, I. & SEKIGUCHI, K., 1968. Some properties of an adenine-rich polynucleotide fragment from the avian reovirus. *Biochemical and Biophysical Research Communications*, 30, 95–99.

LOENING, U. E., 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. *Biochemical Journal*, 102, 251–257.

LOH, P. C. & SHATKIN, A. J., 1968. Structural proteins of reoviruses. *Journal of Virology*, 2, 1353–1359.

MACPHERSON, I. & STOKER, M., 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology*, 16, 147–151.

MELNICK, J. L., 1975. Taxonomy of viruses 1975. *Progress in Medical Virology*, 20, 208–211. Basel: Karger.

OELLERMANN, R. A., 1970. Plaque formation by African horsesickness virus and characterization of its RNA. *Onderstepoort Journal of Veterinary Research*, 37, 137–144.

OELLERMANN, R. A., ELS, H. J. & ERASMUS, B. J., 1970. Characterization of African horsesickness virus. *Archiv für die gesamte Virusforschung*, 29, 163–174.

SCHERRER, K. & DARNELL, J. E., 1962. Sedimentation characteristics of rapidly labelled RNA from HeLa cells. *Biochemical and Biophysical Research Communications*, 7, 486–490.

SHAPIRO, A. L., VIÑUELA, E. & MAIZEL, J. V. Jr, 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochemical and Biophysical Research Communications*, 28, 815–820.

SHATKIN, A. J. & SIPE, J. D., 1968. Single-stranded adenine-rich RNA from purified reoviruses. *Proceedings of the National Academy of Sciences of the United States of America*, 59, 246–253.

SMITH, R. E., ZWEERINK, H. J. & JOKLIK, W. K., 1969. Polypeptide components of virions, top component and cores of reovirus type 3. *Virology*, 39, 791–810.

STUDIER, F. W., 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *Journal of Molecular Biology*, 79, 237–248.

VERWOERD, D. W., ELS, H. J., DE VILLIERS, ETHEL-MICHELE & HUISMANS, H., 1972. Structure of the bluetongue virus capsid. *Journal of Virology*, 10, 783–794.

VERWOERD, D. W. & HUISMANS, H., 1969. On the relationship between bluetongue, African horsesickness and reovirus: Hybridization studies. *Onderstepoort Journal of Veterinary Research*, 36, 175–180.

VERWOERD, D. W., OELLERMANN, R. A., BROEKMAN, J. & WEISS, K. E., 1967. The serological relationship of South African bovine enterovirus strains (Ecbo SA-I and -II) and the growth characteristics in cell culture of the prototype strain (Ecbo SA-I). *Onderstepoort Journal of Veterinary Research*, 34, 41–52.

WEBER, K. & OSBORN, MARY, 1969. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry*, 244, 4406–4412.