REVIEW ARTICLE

Variation in Orbiviruses

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INTRODUCTION

The name orbiviruses was proposed by Borden et al. (1971) to describe a group of arthropod-borne viruses which, by their physico-chemical properties and characteristic appearance by electron microscopy, were sufficiently distinct to form a new taxonomic group. Unlike other arboviruses, these viruses were either less susceptible or resistant to the action of diethyl ether or deoxycholate. The name reflected the especially large doughnut-shaped capsomeres seen on the surface of virus particles by electron microscopy. The orbiviruses included pathogenic agents of man (Colorado tick fever), domestic animals (bluetongue of sheep and African horse sickness), native animals (epizootic haemorrhagic disease of deer) and many other viruses not yet linked with disease.

Verwoerd (1969) isolated double-stranded RNA from purified bluetongue virus and further characterization showed that it consisted of ten fragments similar to but not identical with double-stranded RNA segments isolated from reovirus (Verwoerd et al. 1970). Verwoerd (1970) proposed that certain viruses, by their morphology and in some cases by their RNA composition, should be considered as potential members of a new group of double-stranded RNA viruses named diplornaviruses. Viruses with double-stranded RNA genomes consisting of from two to twelve fragments have been isolated from vertebrate, invertebrate and bacterial hosts and from higher plants and fungi (see Wood, 1973, for review). The International Committee for Taxonomy of Viruses decided that the family Reoviridae should encompass those viruses which (a) possess genomes consisting of several (generally 10 to 12) segments of double-stranded RNA with mol. wt. ranging from \(2 \times 10^6\) to \(3 \times 10^8\), all segments being encapsidated within a single virus particle and (b) possess a quasi-spherical capsid, 60 to 80 nm in diam., which exhibits icosahedral symmetry (Fenner, 1976). Four genera were established: reoviruses, orbiviruses, the cytoplasmic polyhedrosis viruses and the plant reovirus group, with the provisional inclusion of the infantile diarrhoea viruses (rotaviruses) as a separate genus (Fenner, 1976).

The serological groups of orbiviruses

The viruses within the genus orbivirus have been subdivided on the basis of their serological reactions. Viruses which share common complement fixing antigens are grouped under the name of the first virus isolated in that group. Viruses within each group are distinguishable by serum-neutralization tests. The diverse isolation of orbiviruses has led to a problem in terminology. Most were isolated as by-products in programmes to isolate and identify viruses as the causative agents of known arthropod transmitted diseases. The identification of arboviruses is often made at the World Reference Centre for Arthropod-borne Viruses at the Yale Arbovirus Research Centre, New Haven, U.S.A. New viruses are given names and are registered in the Catalogue of Arthropod-borne Viruses of the world (Berge, 1975). In this way most orbivirus sero-groups are named from the first member isolated and consist of viruses with different names (Table 1). The bluetongue and horse
Table 1. Orbivirus serological groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Type</th>
<th>Prototype strains*</th>
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<tbody>
<tr>
<td>A</td>
<td>1 to 9</td>
<td>African horse sickness</td>
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<tr>
<td>B</td>
<td>1 to 20</td>
<td>Bluetongue</td>
</tr>
<tr>
<td></td>
<td>21 to 28</td>
<td>Epizootic haemorrhagic disease of deer</td>
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<tr>
<td></td>
<td>29</td>
<td>Eubenangee</td>
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<tr>
<td></td>
<td>30</td>
<td>Pata</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>Tilligerry</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>Ibaraki</td>
</tr>
<tr>
<td>C</td>
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<td>Colorado tick fever</td>
</tr>
<tr>
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</tr>
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<td></td>
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<td>Wad Medani</td>
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<td>H</td>
<td>1</td>
<td>Warrego</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mitchell River</td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>Wallal</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mudjinbarry</td>
</tr>
<tr>
<td>J</td>
<td>1 to 5</td>
<td>Equine encephalosis‡</td>
</tr>
</tbody>
</table>

Ungrouped viruses

* As listed in catalogue of arboviruses (Berge, 1975 and additions) except where indicated.
† Swanepoel & Blackburn (1976).
‡ Erasmus et al. (1970).
§ Fabiyi et al. (1975).

sickness groups were built up by workers isolating strains of these viruses which gave distinguishable neutralization reactions but shared common complement fixing antigens. The use of names to describe members of most of the orbivirus sero-groups and type numbers to describe the bluetongue and horse sickness groups obscures the fact that the groups are composed of viruses related by use of the same serological tests. The situation is best illus-
trated by the use of individual names for members of the epizootic haemorrhagic disease of
deer (EHD) sero-group and the Eubenangee sero-group, for viruses which have been shown
(Borden et al. 1971; Moore & Lee, 1972) to form part of the bluetongue sero-group. Because
the EHD and Eubenangee sero-groups were established before the relationships with blue-
tongue virus were recognized and also because of the reluctance to type viruses as bluetongue,
especially for the Eubenangee viruses which have not yet been linked with disease, the
suggestion of three groups of viruses within the bluetongue virus sero-group has been
maintained.

The method of classification of the bluetongue virus and horse sickness virus types has
precedence in that they were the first orbivirus sero-groups in which different virus types
were recognized. Since it will be important ultimately to recognize the clustering of viruses
into groups based on their ability to interact genetically, in addition to their serological
reactions, a uniform terminology should be used to describe the orbivirus groups. A system
based on the use of Orbivirus group A, B, C, etc. to cluster the established sero-groups and
of numbers for the sero-types within each group (Table 1) would avoid confusion. The
allocation of letters to major sero-groups follows as far as possible the isolation date recorded
in the International Catalogue of Arboviruses. The major groups are easily recognized in
that African horse sickness is designated A and bluetongue virus B and existing types have
been maintained within these groups. This method is consistent with established practice
within the reoviruses, another genus in the family Reoviridae. Reoviruses are designated
by type numbers and the strain identified. There would be reluctance to accept, for example,
that bluetongue virus type 8 should be referred to as orbivirus B8 and the prototypes are
referred to where possible to avoid reference to a coding system. Nevertheless it is important
to halt the proliferation of virus names. As the orbiviruses become more widely studied,
the comparison between viruses with trivial names will obscure the more meaningful inter-
relationships which could emerge. The method leaves room for combining groups into a
higher classification or for addition of new groups. Viruses not assigned to serological groups
(Table 1) are listed as ungrouped in the International Catalogue of Arboviruses.

Immunological variation

Immunological variation has long been recognized with the virus of bluetongue. The first
description of the disease was given by Hutcheon in 1881 and Theiler suggested in 1906 that
the causative agent was a virus (review Howell, 1963). In 1908, Theiler immunized sheep
by injection of a mild strain of virus which had been serially passaged in sheep. The Theiler
strain (now recognized as type 4) was replaced by the Veglia strain in 1927 since it was
thought that the Theiler strain might have been too attenuated to be a good vaccine. Con-
tinued failure of the Veglia vaccine led to the re-introduction of the Theiler strain in 1938.
Despite evidence that it failed to provide adequate immunity the vaccine continued to be
used for many years. Over 50 million doses of the monovalent Theiler vaccine were issued
over a period of 40 years.

Early investigation into breaks in the immunity after immunization with the monovalent
vaccine failed to appreciate the possible existence of a plurality of virus strains. Neitz (1948)
was the first to recognize antigenically different types and to provide evidence for variation
in virulence among different strains. A polyvalent attenuated live vaccine was introduced
and its composition altered from time to time by addition of attenuated strains which had been
isolated from vaccinal breakdowns, while others were withdrawn in the belief that they were
no longer active in the field. While workers were fully aware of the immunological variation
among strains of bluetongue virus no techniques were available to permit assessment of
these characteristics. Kipps (1956), in a series of cross complement fixation tests with six
bluetongue virus strains, demonstrated a common antigen. Howell (1970) confirmed the
existence of a group-reactive complement fixing antigen and classified 244 naturally occurring bluetongue virus strains into 16 immunological groups by a plaque reduction test in tissue culture. South African workers found the test specific but other workers report cross-neutralization between types (Thomas et al. 1976). Many of the bluetongue strains isolated in Kenya are neutralized to varying degrees by up to three typing sera (F. G. Davies, personal communication).

The literature can be interpreted as suggesting an extended bluetongue virus sub-group consisting of at least 32 viruses (Table 1). The relationship between bluetongue and epizootic haemorrhagic disease of deer (EHD) seems now to be established, if not through the strains which have been isolated in North America, then probably through strains isolated in Nigeria. While much work has been done to separate the viruses in North America, those isolated in Nigeria share common complement fixing antigens with EHD isolated in U.S.A. and with bluetongue virus strains from South Africa (Moore & Lee, 1972). The virus Pata isolated in the Central African Republic shares complement fixing antigens with EHD and also with an Australian orbivirus Eubenangee (Borden et al. 1971). Evidence of cross-reactions between Eubenangee, Tilligerry and bluetongue viruses was found in complement fixation tests in which antigen-antibody-complement mixtures were incubated at 4 °C overnight but not by using an incubation period of 37 °C for 30 min which is routinely applied to bluetongue virus strains (B. M. Gorman, unpublished results). The tests were not conclusive but tended to substantiate the claim of Borden et al. (1971) that the viruses form part of the same group. None of the viruses Pata, Tilligerry and Eubenangee is known to cause disease resembling bluetongue in animals. By contrast Ibaraki virus, which produces clinical bluetongue in cattle but not in sheep, is also related to EHD virus (Campbell et al. 1978). A large number of viruses make up a bluetongue virus serological complex but the exact relationships between them at the molecular level remain to be determined.

The presence of a divided genome and the demonstration of genetic reassortment between orbiviruses (Gorman et al. 1978) suggests that the division of orbiviruses into clearly distinct serological groups may not be possible. Antigenic determinants giving rise to low level cross reactions between two viruses in different serological groups may not be shared by all members of each group.

The structure of orbiviruses

Morphology of orbiviruses

By electron microscopy, Studdert et al. (1966) showed that negatively stained bluetongue virus particles were characteristically 53 nm in diam. with no evidence of an enveloped particle. They suggested that the virus was icosahedral with 92 capsomeres and was similar to reoviruses. Owen & Munz (1966) described icosahedral particles with 92 capsomeres, 60 nm in diam. and fewer enveloped particles 100 nm in diam. Evidence for an outer envelope was also presented by Bowne & Jones (1966) in a study of virus particles in the salivary glands of Culicoides variipennis. Els & Verwoerd (1969) suggested that what other workers had represented as enveloped particles were cellular membranes occasionally wrapped around one or more particles. These structures, called pseudo-envelopes, were removed by treatment with Tween-80 and ether without loss of infectivity. They described a virus particle 55 nm in diam. with 32 capsomeres in contrast to the 92 suggested by other workers. No evidence was found of a double layer capsid similar to that present in reoviruses.

Verwoerd et al. (1972) showed that two polypeptides form a diffuse protein layer surrounding the nucleocapsid of bluetongue virus and obscure the arrangement of structural units in the nucleocapsid. This observation solved the discrepancy between reports on the structure and size of the virion. The particles, 55 nm in diam. with 32 clearly discernible
capsomeres, observed by Els & Verwoerd (1969), represented subviral particles while the ill-defined particles represented the virion. The structure of the bluetongue virus capsid (Verwoerd et al. 1972) is similar to that of reovirus both in size of the complete particles and in the possession of a double layer. However, the assembly of the viruses is different. In reovirus the outer layer consists of structural units arranged regularly and an inner capsid that has an ill-defined structure. In bluetongue virus the outer layer is diffuse and unstructured whereas the inner layer consists of conventional pentamer-hexamer morphological units of capsomeres. Martin & Zweerink (1972) confirmed that bluetongue virus (B10) consisted of a clear nucleocapsid structure with 32 capsomeres and a diam. of 63 nm surrounded by two polypeptides which gave the complete virion a diam. of 69 nm.

In view of the discrepancy between reports of the structure of bluetongue virus before the correct structure was revealed by Verwoerd et al. (1972), the reported structure of other orbiviruses (see review by Joklik, 1974) should be viewed with some caution. The reports could need re-interpretation in that nucleocapsids rather than complete virions may have been described. Lecatsas & Gorman (1972) demonstrated the existence of an outer layer surrounding the nucleocapsids of seven orbiviruses, five of which had been described previously (Corriparta, Eubenangee, Warrego, Mitchell River and D’Aguilar) on the assumption that the nucleocapsid represented the complete virion. The large range of sizes and the differences in morphology reported for orbiviruses may not be significant. Verwoerd et al. (1972) reported that bluetongue virus particles which had lost one polypeptide had discernible capsomeric structure but with relatively poor definition of the capsomeres. It is difficult to interpret which particles were measured in many studies of orbivirus morphology. It seems likely though that all orbiviruses have a morphology similar to Wallal virus (Fig. 1).

**RNA composition**

The first indication that bluetongue virus contained RNA as its genetic material was derived from cytochemical studies using acridine orange (Livingston & Moore, 1962). Clearly defined orange-red inclusion bodies were observed in the cytoplasm from 4 h p.i. Verwoerd (1969) demonstrated the double-strandedness of the bluetongue virus genome.
Table 2. Separation by PAGE of double-stranded RNA genomes of orbiviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Range in mol. wt. (× 10⁻⁶) of genome segments 1-10</th>
<th>Mol. wt. (× 10⁻⁶) of total genome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>2.5-0.25</td>
<td>11.6</td>
<td>Bremer, 1976</td>
</tr>
<tr>
<td>B10</td>
<td>2.7-0.28</td>
<td>13.1</td>
<td>Martin &amp; Zweerink, 1972</td>
</tr>
<tr>
<td>B21</td>
<td>2.6-0.32</td>
<td>11.4</td>
<td>Huismans et al., 1979</td>
</tr>
<tr>
<td>B32</td>
<td>2.7-0.32</td>
<td>12.5</td>
<td>Saito et al., 1978</td>
</tr>
<tr>
<td>B29</td>
<td>2.5-0.39</td>
<td>12.1</td>
<td>Schnagl &amp; Holmes, 1975</td>
</tr>
<tr>
<td>B31</td>
<td>2.6-0.26</td>
<td>11.8</td>
<td>Gorman &amp; Taylor, 1978</td>
</tr>
<tr>
<td>D4</td>
<td>2.5-0.32</td>
<td>11.5</td>
<td>Schnagl &amp; Holmes, 1975</td>
</tr>
<tr>
<td>F1</td>
<td>2.5-0.23</td>
<td>11.6</td>
<td>Gorman, 1978</td>
</tr>
<tr>
<td>G10</td>
<td>3.0-0.46</td>
<td>12.5</td>
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<td>H1</td>
<td>2.4-0.22</td>
<td>11.3</td>
<td>Gorman et al., 1977a</td>
</tr>
<tr>
<td>H2</td>
<td>2.2-0.24</td>
<td>11.0</td>
<td>Gorman et al., 1977a</td>
</tr>
<tr>
<td>I1</td>
<td>2.3-0.22</td>
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<td>Gorman et al., 1978</td>
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<tr>
<td>I2</td>
<td>2.3-0.23</td>
<td>11.3</td>
<td>Gorman et al., 1978</td>
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</tbody>
</table>

by thermal denaturation, by its resistance to ribonuclease degradation and by base composition analysis. Verwoerd et al. (1970) fractionated bluetongue virus RNA into five components by sucrose gradient sedimentation analysis and into ten components by electrophoresis in polyacrylamide gels. By co-electrophoresis of bluetongue virus RNA and reovirus RNA, Verwoerd et al. (1972) showed that the mol. wt. of the segments ranged from 0.30 to 2.50 × 10⁶ with the total mol. wt. for the genome of type 10 being 11.8 × 10⁶. Reports of the separation of the RNA of orbiviruses into ten segments by polyacrylamide gel electrophoresis are summarized in Table 2 and the range of mol. wt. of the genomes of orbiviruses shown to be between 11 and 12.5 × 10⁶. Variations between viruses may reflect the different experimental conditions used by different workers.

Green (1970) isolated RNA from Colorado tick fever virus and centrifuged the RNA and ribonuclease treated RNA in sucrose density gradients. Two peaks were observed in untreated RNA, one at 14S and the other at the 4S region; only a 14S peak was observed in the ribonuclease treated RNA. The 4S peak was assumed to be cellular or serum components not removed by the procedure used for purification of virus. The 14S ribonuclease resistant peak was assumed to be the double-stranded genome of the virus. Ribonuclease-resistant RNA of Kemerovo virus sedimented at 14S (Rosenbergova & Slavik, 1975).

Polypeptide composition

The capsid of bluetongue virus contains four major polypeptides and three minor polypeptides (Verwoerd et al. 1972; Martin & Zweerink, 1972). Using the degrading effect of unbuffered caesium chloride on bluetongue virus, Verwoerd et al. (1972) were able to show that two polypeptides constitute the diffuse layer surrounding the nucleocapsid. An RNA-dependent RNA polymerase was activated by removal of these two polypeptides. Loss of either one or both of the outer polypeptides resulted in a loss of infectivity (Verwoerd et al. 1972). Martin & Zweerink (1972) found that light particles (1.36 g/ml) in caesium chloride contained seven polypeptides and had a specific infectivity about 10- to 100-fold greater than particles (1.38 g/ml) which contained five polypeptides.

The capsid proteins of bluetongue virus apparently exhibit a primary gene product relationship with the genome RNA segments. Based on the assumption that each genome segment is fully transcribed and translated, the relationships between RNA segments and structural proteins are shown in Table 3. Polypeptides 2 and 5 were shown by Verwoerd
Table 3. Location of polypeptides in bluetongue virus capsids and possible relationships with RNA genome segments

<table>
<thead>
<tr>
<th>RNA segment</th>
<th>Polypeptide</th>
<th>Mol. wt. (× 10⁻⁶)</th>
<th>No. per virion*</th>
<th>Location†</th>
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<tr>
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<td>8</td>
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<td>—</td>
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<td>0.5</td>
<td>7</td>
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<td>570</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>—</td>
<td>17†</td>
<td>—</td>
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</table>

* Verwoerd et al. 1972.
‡ Estimated from mol. wt. of RNA segment.

et al. (1972) to form the outer layer. This was confirmed by Martin et al. (1973) who also showed by labelling with radio-iodine that polypeptides 1, 3 and 4 were on the outer surface of the nucleocapsid.

Purified epizootic haemorrhagic disease of deer virus contains four major and four minor polypeptides. The additional minor component of mol. wt. 90000 was located in the outer layer (Huismans et al. 1979). The serologically related Ibaraki virus is reported to be structurally similar to bluetongue virus and contains four major and three minor polypeptides (Saito et al. 1978).

Bremer (1976) found seven polypeptides by PAGE of purified African horse sickness virus. The relative amounts of the four major and three minor components were similar to those found in bluetongue virus but polypeptides 3 and 5 comprised the outer protein layer and polypeptides 1, 2, 4, 6 and 7 the nucleocapsid. Seven polypeptides similar in mol. wt. distribution to those of bluetongue virus were found in purified preparations of Wallal virus but the location of individual polypeptides was not determined (Gorman et al. 1973).

Replication of orbiviruses

In an examination by electron microscopy of cells infected with bluetongue virus, Lecatsas (1968) reported the development of dense inclusion bodies and the appearance of masses of fine filaments in the cytoplasm. Murphy et al. (1971) demonstrated a similar morphology and mode of morphogenesis for bluetongue, EHD, Colorado tick fever, Palyam, Irituia, Chenuda, Tribec, Lebombo and Wad Medani viruses. The viruses developed from a granular or reticular cytoplasmic matrix and with rare exceptions were unenveloped. Filamentous and tubular structures were characteristic associated with the sites of maturation entirely within the cytoplasm. There are numerous essentially similar descriptions of the morphogenesis of other orbiviruses (for review see Joklik, 1974).

After infection, the 10 double-stranded RNA genome segments are apparently transcribed into 10 single-stranded RNA species which have been shown for bluetongue virus to hybridize uniquely with denatured double-stranded RNA (Verwoerd & Huismans, 1969). This RNA transcription activity has also been demonstrated in vitro with purified virus, but only after removal of the surface polypeptides (Martin & Zweerink, 1972; Verwoerd et al. 1972). The relative rates of transcription of the genome segments remain essentially constant throughout the multiplication cycle but genome segment 5 is transcribed at twice the fre-
Fig. 2. PAGE of proteins induced by bluetongue virus type 20 in BHK-21 cells. (a) Virus-infected cells treated with methionine-free Eagle's MEM + 35S-methionine for 15 min at 12 h p.i. (b) As for (a) with removal of 35S-methionine after 15 min and incubation in Eagle's MEM + excess unlabelled methionine for 75 min. Analysis by PAGE by the method of Laemmli (1970).

quency and segment 10 at half the frequency of the remaining segments. This regulation of transcription occurs in vivo and in vitro (Huismans & Verwoerd, 1973) but its significance is not known.

Synthesis of orbivirus proteins in infected cells can be readily observed late in the infectious cycle. The viruses depress host cell protein synthesis (Huismans, 1971) and introduction of radiolabelled amino acids at this stage leads to specific incorporation of the label into virus proteins. Huismans (1979) detected nine polypeptides in cells infected with bluetongue virus type 10. In addition to the seven structural polypeptides of the virion, non-structural polypeptides of mol. wt. 54000 (P5a) and 40000 (P6a) were found. The intra-
cellular proteins induced in BHK-21 cells by bluetongue virus type 20 are shown in Fig. 2 (B. M. Gorman & D. V. Sangar, unpublished data) and numbered according to Huismans (1979). Fig. 2 shows the results of pulse-chase experiments and suggests that no secondary cleavage of virus proteins occurs in orbivirus replication.

Polypeptide 5a appears to be associated with the intracytoplasmic tubular structure characteristic of orbivirus infections (Huismans & Els, 1979) and is synthesized in large quantities throughout the infectious cycle. It has been suggested that the relative excess of polypeptide 5a correlates with the observation that genome segment 5 is transcribed at twice the frequency of the other segments (Verwoerd et al. 1979). The function of the other non-virion polypeptide (P6a) is not known. A protein of mol. wt. 15,000 corresponding to the translation product of genome segment 10 has not been observed in experiments with bluetongue virus type 10 (Huismans, 1979) or type 20 (B. M. Gorman & D. V. Sangar, unpublished data).

Polypeptides equivalent to P5a and P6a have been described in cells infected with EHD virus (Huismans et al. 1979). In addition they describe eight polypeptides in purified virus but found no polypeptide corresponding to genome segment 10. The authors did not suggest a method for processing gene products to allow the generation of ten polypeptides from nine genome segments.

The method of assembly of virus particles is not known. Presumably, like reovirus (Joklik, 1974), the singled-stranded RNA acts as a template for the synthesis of complementary minus strands in the production of doubled-stranded genome segments. It is presumed that single-stranded RNA acts both as messenger RNA and as a template for genome RNA. The method of packaging one copy of each of the genome segments into assembled virions has yet to be explained. While random assembly confers advantages through the increased chance of genetic interactions, the probability of assembling a complete set of ten genes in one virus particle is extremely low. However, specific association of RNA and protein in virus assembly has not been demonstrated although recent evidence by electron microscopy suggests that the genome segments are linked (Foster et al. 1978).

**Correlation of antigenic differences between orbiviruses with molecular structure**

**Heterogeneity in size of RNA segments in closely related viruses**

The use of the technique of electrophoresis of RNA in acrylamide gels can give some indication of the relatedness of orbiviruses. Electrophoretic separation of the genome segments of Warrego and Mitchell River viruses of the Warrego sero-group (H) showed considerable differences in the RNA patterns (Gorman et al. 1977a). Co-electrophoresis of the RNAs of the two viruses revealed 18 bands suggesting that the viruses are distantly related. Only low level cross reactions are found in complement fixation tests and no cross neutralization can be detected between the viruses. However, the serotypes of the Wallal serological group which are indistinguishable by complement fixation tests also showed considerable differences in the electrophoretic separation of their RNAs (Fig. 3; Gorman et al. 1978). Heterogeneity in the sizes of genome segments has been described for serotypes of the Eubenangee serological group (Gorman & Taylor, 1978) and has been observed with RNA segments of bluetongue virus serotypes (B. M. Gorman, unpublished data; H. Huismans, personal communication). It has been suggested that the patterns of separation of virus double-stranded RNA in acrylamide gels can be used to classify viruses. Payne & Rivers (1976) showed heterogeneity in genome segment sizes of cytoplasmic polyhedrosis viruses and it was suggested (Payne et al. 1977) that the electrophoretic profiles were more useful in grouping the viruses than were serological methods. Similar heterogeneity has been shown for segments of the reovirus serotypes (Shatkin et al. 1968; Ramig et al. 1977) and also between strains of reovirus type 3 (R. F. Ramig, personal communication). Analysis
of 17 isolates typed by complement fixation test as Wallal group viruses showed that use of PAGE profiles of RNA alone would be inadequate for classification of these viruses (Gorman et al. 1977b). The basis of the extensive variation in sizes of segments of even closely related isolates (by serum-neutralization test) requires further investigation.

Co-migration of RNA segments indicates similarity in mol. wt. only and does not indicate the relationship between segments. Furthermore, segments containing information coding for equivalent antigenic sites in different viruses may migrate to different positions in PAGE. However, analysis of the genomes of orbiviruses by PAGE suggests diversity which has not so far been recognized in conventional serological tests.

RNA–RNA hybridization

Huismans & Howell (1973) attempted to correlate the antigenic differences with molecular structure by molecular hybridization studies between the RNA of bluetongue virus strains. They studied the relationships between serotypes of bluetongue virus and the differences between virulent and attenuated strains of the same serotype by cross-hybridization of messenger-RNA (mRNA+) with the complementary minus strand of the virus double-stranded RNA (dsRNA). The technique involved isolating $^3$H-labelled mRNA from cells infected with one serotype and hybridizing it with saturating amounts of denatured $^{32}$P-labelled dsRNA from bluetongue virus type 10. The products were analysed by electrophoresis in acrylamide gels. $^{32}$P-activity in the gel showed the location of the original dsRNA since the bulk of the denatured dsRNA recombined to form the original ten dsRNA seg-
ments. A small proportion of the denatured dsRNA hybridized with the $^3$H-labelled mRNA to form a duplex and the position of the $^3$H-label in the gel allowed an assessment to be made of the degree of hybridization. Duplexes from homologous hybridization between single-stranded RNA from bluetongue virus hybridize specifically with the (−) strand of the dsRNA to form ten dsRNA segments indistinguishable in electrophoretic mobility from normal dsRNA segments (Verwoerd & Huismans, 1972). Incomplete hybrids in cross-hybridization experiments can be located by a change in migration in PAGE compared with the original segment.

Cross-hybridization of RNAs of three strains of bluetongue type 4 showed considerable mis-matching in each of the genome segments. No antigenic differences were detected between those strains which were isolated in South Africa in 1901 and 1958 and in Cyprus in 1971 (Huismans & Howell, 1973). Indeed no antigenic differences have been detected between type 4 strains isolated at various times in South Africa nor between these and strains isolated in Israel in 1954 (Howell & Verwoerd, 1971). The altered base sequences suggested by the RNA hybridization results need not necessarily be reflected in alterations to the antigenic sites on virus proteins. It is more likely, however, that the apparent stability of antigenic determinants is due to the failure of conventional serological tests to detect minor variation.

In cross-hybridization experiments between serotypes of bluetongue virus, Huismans & Howell (1973) found incomplete homology in most of the duplexes examined and in many cases no duplexes were found. Cross-hybridization of mRNA from eight serotypes with dsRNA from bluetongue type 10 suggested that complete hybrids were formed between segments 5 in five serotypes and minor alterations occurred in three serotypes. By contrast, segments 2 and 6 of type 10 did not hybridize with the corresponding segments of any other type. Absence of hybrids between some other segments was also observed (Huismans & Howell, 1973).

Failure to demonstrate hybridization between certain segments indicates significant differences in nucleic acid homology and could suggest that different rather than altered segments are present in some viruses. The demonstration of genetic reassortment between orbiviruses (Gorman et al. 1978) would support such an interpretation. However, the method chosen by Huismans & Howell (1973) eliminated partial hybrids in the comparative analysis. Recent findings of heterogeneity in size of genome segments of related viruses suggest the possibility of formation of partial hybrids between segments of different sizes. Thus the reported lack of homology between certain segments of bluetongue virus types requires further examination.

Cross-hybridization of RNA of bluetongue type 10 and African horse sickness type 3 showed less than 5% homology between them (Verwoerd & Huismans, 1969). Similarly, little homology was found between the nucleic acids of epizootic haemorrhagic disease of deer virus (New Jersey strain) and bluetongue virus type 10 (Huismans et al. 1979). Less than 10% cross-hybridization was observed and no hybrid double-stranded RNA segments were identified.

Analysis of virus polypeptides

The surface polypeptides of orbiviruses are likely to be involved in neutralization reactions with antibody. De Villiers (1974) compared the molecular sizes of capsid polypeptides of serotypes of bluetongue virus with those of bluetongue virus type 10 and found significant differences between serotypes. The mol. wt. of polypeptide 2 differed in nine of the eleven serotypes compared with type 10. The mol. wt. of polypeptide 5 differed in four out of eleven serotypes and two of these were strains for which a significant difference in mol. wt. was not detected in polypeptide 2. Huismans (1979) used homologous and heterologous antisera to precipitate proteins from virus-infected cells and compared the immune precipitates by
Polypeptide 2 of bluetongue virus was precipitated only with homologous serum suggesting that this polypeptide contains the type-specific antigen in bluetongue virus.

Genetic reassortment as a mechanism for variation in orbiviruses

The presence of a segmented genome in orbiviruses is ideal for interchange of genetic information. In cells infected simultaneously with two viruses the possibility exists for interchange of RNA segments with the consequent exchange of antigens and emergence of new types. The use of temperature-sensitive mutants of reoviruses has shown that wild-type virus was formed with high frequency at the non-permissive temperature in cells infected with certain combinations of mutants (Fields & Joklik, 1969). The phenomenon was thought not to result from genetic recombination involving breakage and reunion of covalent bonds but was due to reassortment of RNA segments (Joklik, 1974). Recent evidence shows that the recombinants arise by physical reassortment of genome segments between parent reoviruses (Sharpe et al. 1978).

High frequency recombination between temperature-sensitive mutants, suggesting genetic reassortment, has been shown for bluetongue type 10 (Shipham & De La Rey, 1976). Similar high frequency recombination has been demonstrated between temperature-sensitive mutants of Wallal virus and also between mutants of the serotypes Wallal and Mudjinbarry viruses (Gorman et al. 1978). Wild-type plaques appearing at the non-permissive temperature after crossing a Mudjinbarry mutant and a Wallal mutant were analysed for RNA content by PAGE. The electrophoretic mobilities of segments 1, 7, 8 and 9 of the parent viruses are indistinguishable by this method. Two recombinants with RNA patterns differing from each parent were detected in 60 plaques selected. One derived segment 5 from Mudjinbarry virus and the remaining segments apparently from Wallal virus; the other derived segments 4 and 6 from Mudjinbarry virus. More detailed analysis of recombinant and parent viruses by the technique of oligonucleotide mapping (Walker & Melzer, 1978) has revealed that each recombinant also contained Mudjinbarry segment 1. It is apparent that segment 1 of the Wallal virus mutant contained the temperature-sensitive lesion and the appearance of wild-type progeny at the non-permissive temperature was due to its replacement by segment 1 of the Mudjinbarry mutant (P. J. Walker, personal communication). The result shows that high frequency recombination in orbiviruses occurs because of interchange of complete genome segments. The isolation of recombinants containing more than one segment derived from each parent suggests random reassortment of unlinked genome segments.

The demonstration of genetic reassortment between serotypes of an orbivirus group suggests that natural variants may be produced in simultaneous infection of hosts with two or more viruses. The possibility of simultaneous infections is high in situations where the distribution of antigenic types is widespread in insect and vertebrate populations. Howell et al. (1970) reported the simultaneous isolation of numerous antigenic types of bluetongue virus from a single flock of infected sheep. Davies (1978) reported continuous challenge of a group of sentinel cattle near Nairobi, Kenya, by 19 strains of bluetongue virus. He found that the 19 strains were active, during the same 5-year period, throughout Kenya. Barber & Jochim (1975) isolated two serotypes of EHD and one of bluetongue virus from the same herd of cattle in Colorado, U.S.A. A study of viruses isolated from Culicoides spp. within 11 days near Charleville, Australia, revealed at least six electrophoretic variants which were classified as Wallal virus by conventional serological tests (Gorman et al. 1977b).

Any assessment of the likelihood of multiple infections must take into consideration Howell's observation in 1966 that examination of numerous specimens from naturally infected sheep and cattle over many years failed to show the presence of more than one strain of virus in a single animal. It could be significant that it was possible to demonstrate
simultaneous circulation of more than one strain of bluetongue virus in an animal experimentally infected by the intravenous inoculation of polyvalent vaccine (Howell & Verwoerd, 1971).

CONCLUSION

The establishment of serological groups has been important in the assessment of relationships between viruses and in the development of concepts of evolution of viruses. However, the usefulness of the results of serological tests in estimating diversity in virus populations is limited. The application of serum-neutralization tests in defining orbivirus serotypes, which correlate with cross-protection tests in infected animals, has obvious importance in the prevention of disease, yet the test measures the relatedness between antigenic determinants carried by possibly one protein (Huismans, 1979) for which the genetic information is provided by one of the ten genome segments. The detection of shared antigens in complement fixation tests has application in defining groups of orbiviruses but is restricted to comparing certain antigenic sites. The technique of hybridization of RNAs of viruses provides a more sensitive method for comparing their genetic composition either by comparative hybridization of complete genomes (Verwoerd & Huismans, 1969) or by analysis of hybrids formed between RNAs of related viruses (Huismans & Howell, 1973). Electrophoretic analysis of RNA genome segments suggests a diversity not previously recognized within orbivirus serological groups. The significance of different electrophoretic migration of RNA segments has yet to be established but analysis of electrophoretic patterns of proteins of a variety of organisms has established that natural populations are genetically heterogeneous (Lewontin, 1976).

The significance of genetic reassortment in generating diversity can only be established by estimating gene frequencies within orbivirus populations. The estimate of genetic similarity and diversity between virus isolates within the same population will also define the degree of divergence necessary to exclude genetic interaction between virus populations and so define species within orbiviruses. Comparative analysis of each of the ten genes and their function is needed to understand the genetic basis of diversity within orbiviruses.

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REFERENCES


Review: Variation in orbiviruses


