

Comparative Aspects of Togaviruses

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INTRODUCTION

In 1970 the Vertebrate Virus Subcommittee of the International Committee on Nomenclature of Viruses proposed the name togavirus (from the latin *toga* – a cloak) to cover arboviruses having taxonomic characters like those of the serological groups A and B (Andrewes, 1970). The name ‘arbovirus’ should be used in a purely biological sense, i.e. for viruses having a biological cycle in both arthropods and vertebrates; it is endowed with ecological but not with structural significance. During the last few years, a considerable amount of information concerning the structure and multiplication of these agents has been accumulated, confirming that alpha- and flavoviruses, as they are named now (Wildy, 1971), are sufficiently different from other virus groups to justify this classification.

The arthropod-borne members of the togavirus family are relatively small spherical RNA-viruses which are sensitive to treatment with organic solvents or mild detergents. The virion is constructed of a spherical core which is wrapped into an envelope carrying projections on its surface. The genome consists of a single-stranded RNA which is infectious when extracted and assayed under appropriate conditions. Since arthropod transmission has been found for members of structural groups other than togaviruses it conceivably is not correlated with virus architecture; certain members of the picorna-, reo- and rhabdovirus group of viruses are transmitted by blood-sucking arthropods while most of them are not. Consequently non-arthropod-borne (nonarbo) viruses of the togavirus family, too, had to be expected.

Rubella virus (RU) of man was one of the first nonarbo togavirus candidates (Holmes & Warburton, 1967); subsequently lactic dehydrogenase virus (LDH) of mice (Melnick, 1969) and the causative agents of equine arteritis (EA), bovine viral diarrhoea – mucosal disease (VD) and hog cholera (HC) have been added (Horzinek, Maess & Laufs, 1971). A plant virus (carrot mottle virus) has also been shown to possess structural characteristics of this family (Murant, 1971).

The apparent widespread distribution of togaviruses in nature and the anticipation that other yet unclassified or newly isolated animal viruses will be identified as belonging to this family motivates a comparative review. Therefore similarities and differences to other isometric enveloped RNA-viruses ≤ 100 nm in diameter, namely orthomyxo-, Bunyamwera supergroup (Uukuniemi virus is discussed together with the members of the Bunyamwera supergroup), corona-, arena- and leukoviruses will be considered with the aim of obtaining distinguishing criteria which might be useful for identification and classification of togaviruses. Whenever possible the references have been minimized by quoting general reviews; a paper comparing the properties of individual members of the togavirus family is in the press (Horzinek, 1973).

VIRUS MORPHOLOGY AND ANATOMY

Shape

Electron microscopy of negatively stained preparations of alpha-, flavo- and nonarbo togaviruses revealed predominantly spherical particles consisting of an isometric core (equivalent to the nucleocapsid) and a more or less tightly fitting envelope. The latter is responsible for the observed variation in virion diameter, being non-rigid and delicate, as evidenced by deviations from the circular particle silhouette and by 'bleb' artefacts in negatively stained preparations – tail-shaped protrusions which are common also in other enveloped viruses and may give them a tadpole-like appearance. In addition, the coefficients of variation (standard deviation/mean value) for the diameters of whole particles are invariably greater than those for the nucleocapsids and may depend from the situation of an individual particle with respect to the thickness and border of the stain droplet (Horzinek & Mussgay, 1969).

When compared with particles of the orthomyxo-, corona- and arenaviruses, however, togavirions show relatively little deviation from spherical shape and variation in diameter. In freshly isolated influenza virus strains filamentous forms of great length occur and it is possible that the filamentous rather than the spherical morphology is usual in naturally occurring infections (Webster & Laver, 1971). Elongated togavirus particles containing internal components of about the same width and staining density as the nucleocapsid have been identified in infected cells; in these cases an aberrant assembly of capsid subunits may have occurred such as is known for other naked viruses with cubic symmetry (e.g. rod-shaped forms of papovaviruses – Horne & Wildy, 1961).

On the other hand, tubular alphavirions containing more than one spherical nucleocapsid (Tan, 1970) and multiploid 'giant forms' containing up to six cores per virion (Simon, 1972) have also been described. Coronavirions (Bradburne & Tyrrell, 1971) and arenavirions (Murphy *et al.* 1970; Lehmann-Grube, 1971) appear extremely variable in outline but filamentous forms have not been reported. Leukoviruses are rather uniform in size and shape possibly because of the stabilizing effect of the rigid icosahedral core structure (Nermut, Frank & Schäfer, 1972).

Envelope

The togavirion envelope may display a smooth surface (EA, VD, HC and LDH viruses) or may carry more or less prominent projections. Alphaviruses and RU virus present themselves as fuzzy spheres, the appearance being caused by a fine, netlike coating probably consisting of loops (Horzinek & Mussgay, 1972; Horzinek, 1973). A similar fringe of poorly defined surface structures can be discerned in Bunyamwera supergroup particles (Holmes, 1971; Pettersson *et al.* 1971). The projections of togaviruses differ morphologically from the rod-shaped spikes of myxoviruses, the club- or petal-shaped coronavirus projections, the cup-shaped surface structures of oncornaviruses and the distinct granular structures described for the envelope of arenaviruses. Unit membrane characteristics have been reported for the envelope of togaviruses in thin sections as well as for the other viruses reviewed here.

Internal structure

A structural detail essential for the recognition of a virus as a member of the togavirus family is the construction of the nucleocapsid. During the last years evidence for its cubic symmetry has accumulated. In alphaviruses radial internal patterns (Simpson &

Hauser, 1968) and polygonal core subunits with an electron-dense centre (Osterrieth, 1968) have been observed within the virion, indicating an icosahedral rather than a helical architecture of the virus nucleocapsid. Horzinek & Mussgay (1969) have demonstrated that the nucleocapsid consists of 32 ring-shaped morphological subunits which are arranged within a surface lattice of the $T = 3$ class. All these workers agree upon a minimum size of 12 nm for the individual capsomere. Cubic symmetry of the alphavirus nucleocapsid has been recently confirmed by Brown, Waite & Pfefferkorn (1972) using the freeze-etching technique; assuming a diameter of 7 nm for the morphological core subunit, however, these authors arrive at an estimate of 92 morphological subunits and a T-9 arrangement. Also, core particles released from flavoviruses after spontaneous envelope disruption (Abdelwahab *et al.* 1964) or osmotic shock treatment (Matsumura, Stollar & Schlesinger, 1971) showed features which are suggestive of cubic symmetry.

Of the nonarbo togaviruses, RU virus particles after sodium deoxycholate treatment revealed cores with angular silhouettes (Holmes, Wark & Warburton, 1969), thereby suggesting an icosahedral nucleocapsid symmetry. The construction of VD virus cores out of spherical subunits and its probable cubic symmetry have been discussed (Maess & Reczko, 1970) and in thin sections through cells infected with HC virus – which is antigenically related to VD virus – hexagonal contours of the cores have repeatedly been observed (Scherrer *et al.* 1970). Also, negatively stained LDH virus in some of the electron micrographs published by Bladen & Notkins (1963) displays the shape of a regular hexagon. In a comparative analysis of the viruses of EA, RU, VD and HC, Horzinek *et al.* (1971) have demonstrated isometric cores in all of them; penetration of the negative stain through the envelope or its removal could be achieved by pretreatment of the purified preparations with urea, saponin, sodium deoxycholate or NP 40.

The alternative type of nucleocapsid symmetry with a helical arrangement of the subunits has been known for the orthomyxoviruses for a long time. When applying the NP 40 procedure to Bunyamwera supergroup viruses (Pettersson *et al.* 1971; Saikku *et al.* 1971), elongated ribonucleoprotein strands could be visualized. Coronaviruses also appear to contain helically symmetric nucleocapsids (Bradburne & Tyrrell, 1971). The internal construction of leukoviruses appears more complex; the envelope encloses the 'core' which presumably possesses cubic symmetry (Nermut *et al.* 1972); however, this constituent is certainly not equivalent to the core of togaviruses since it contains a further elongated structure – the nucleocapsid proper – for which many workers assume a helical symmetry (Sarkar, Nowinsky & Moore, 1971).

Within the nucleocapsid of togaviruses an electron-lucent centre (central core component) has been demonstrated in negatively stained preparations and in thin sections, giving a ring-shaped appearance to the densely staining core. Bunyamwera supergroup viruses may occasionally have a similar appearance but mostly show a finely filamentous central area in sections (Holmes, 1971). In the interior of myxovirus particles dense strands can be discerned when filamentous virions are sectioned longitudinally, whereas in particles with circular profiles round dense spots appear which correspond to cross sections of the nucleocapsid (Compans & Dimmock, 1969). Similar dot patterns have been observed in coronaviruses (Bradburne & Tyrrell, 1971; Cunningham, Spring & Nazerian, 1972) where they may have the same significance. Arenaviruses contain characteristic fine granules, most probably host cell-derived ribosomes (Pedersen, 1971); this structural detail is reflected by the name chosen for the group (from *arenosus*, (Latin) = sandy; Rowe *et al.* 1970). Leukoviruses show three concentric ring structures in thin sections, corresponding to the envelope, the core and the nucleoid (Nermut *et al.* 1972).

PHYSICAL PARAMETERS

Size

The diameter values for arthropod-borne and nonarbo togaviruses as determined by measurements from electron micrographs have been reviewed by Mussgay (1964) and Horzinek (1973). It can be seen from Table 1 that togaviruses are the smallest lipid-containing animal viruses so far described. EA and RU virus, together with most alphaviruses, have somewhat greater diameters than VD, HC virus and members of the flavovirus group; the nucleocapsids measure 30 to 40 nm and 20 to 30 nm across, respectively. Leukovirus cores are about twice that size, ranging from 74 to 80 nm in diameter (Nermut *et al.* 1972). The smaller size of togaviruses as compared with other enveloped RNA-viruses is reflected by their significantly lower sedimentation coefficients (Table 1).

Buoyant density

The buoyant densities of nonarbo togaviruses fall between 1.15 and 1.20 g/ml, when centrifuged for short periods in preformed potassium salt and sucrose gradients. Values up to 1.24 g/ml have been obtained for alpha- and flavoviruses and for nonarbo togaviruses after isopycnic centrifugation in CsCl solutions (Horzinek, 1973). For the apparent variation in the data published, the water extrusion phenomenon (McCombs & Rawls, 1968) and salt-induced virion breakdown (Aaslestad, Hoffman & Brown, 1968) may have been responsible. Similar deleterious effects of concentrated salt solutions have been reported for members of the other groups under comparison. Viruses of the Bunyamwera (Pettersson *et al.* 1971), corona (Bradburne & Tyrrell, 1971) and arena group (Pedersen, 1971) possess densities between 1.18 and 1.20 g/ml and orthomyxoviruses band at 1.22 g/ml in sucrose gradients (Robinson & Duesberg, 1968). Low values similar to those determined for nonarbo togaviruses have been reported for leukoviruses, which accumulate at densities between 1.14 and 1.22 g/ml (Vigier, 1970; Robinson & Duesberg, 1968).

CHEMICAL COMPOSITION

Ribonucleic acid

Togaviruses are the only enveloped RNA viruses which yield infectious RNA, mild detergent treatment being sufficient for extraction. As discussed by Baltimore (1971), the infectivity of a virus RNA suggests that it must be of the same strand as the mRNA; a polymerase then cannot exist within the virion or at least it does not serve an obligate role for the initiation of infection. The genome of togaviruses is essentially single-stranded, although a certain minimal degree of secondary structure has been indicated for members of the alphagroup (Sprecher-Goldberger, 1964; Sreevalsan *et al.* 1968; Stern & Friedman, 1969). It consists of a continuous length polynucleotide with a mol. wt. of about 3 to 4×10^6 . In comparative analyses alphavirus and EA virus RNAs appeared to sediment slightly faster than flavovirus and VD virus RNAs (Boulton & Westaway, 1972; M. Horzinek & V. Moennig, unpublished results). Virion RNA (sedimenting at about 40 S) could be converted into a single-stranded RNA species with a sedimentation coefficient of about 26 S by, for example, urea treatment; the degradation product appears to consist of half-size RNA pieces (Boulton & Westaway, 1972) which cannot be identical in base sequence because the protein coding information required during replication is considerably in excess of that supplied by 2×10^6 daltons of RNA. The 26 S RNA, occurring naturally in infected cells or obtained after denaturation, retains about 1 to 10 % of the infectivity of virus RNA (Sreevalsan *et al.* 1968).

In isolated nucleocapsids, alphavirus RNA is accessible to RNase since treatment with

Table 1. *Diameters and sedimentation coefficients of togaviruses and other enveloped RNA viruses*

Virus group	Average diameter (nm)	Range (nm)	References	Sedimentation coefficient (S)	References
Toga					
Alphaviruses	60	38-89	Horzinek, 1973	280	Boulton & Westaway, 1972
EA, RU				240	Horzinek, 1973
Flavoviruses	45	30-55		205	Boulton & Westaway, 1972
VD, HC				> 100	Horzinek, 1973
Orthomyxo	100	Filamentous forms up to 4000	Compans & Choppin, 1971	600- > 800	Hoyle, 1968
Bunyamwera	90	60-130	Holmes, 1971	450	Pettersson <i>et al.</i> 1971
Corona	—	80-160	Bradburne & Tyrrell, 1971	390	Hierholzer <i>et al.</i> 1972
Arena	120	50-350	Rowe <i>et al.</i> 1970 Murphy <i>et al.</i> 1970	470-500	Pedersen, 1971
Leuko	100	—	Vigier, 1970	500-655	Vogt, 1965

this enzyme resulted in a reduction of their sedimentation coefficient and RNA content and conversion of the RNA into acid soluble fragments (Acheson & Tamm, 1970; Kääriäinen & Söderlund, 1971). The genome of orthomyxoviruses exists as several discrete pieces; as much as 7 to 10 polynucleotide species have been found in influenza virus. Assuming that each virion contains one molecule of each species and that each species is part of the virus genome, a maximum total mol. wt. of approximately 5×10^6 has been estimated (Lewandowski, Content & Leppla, 1971), which is about twice the figure discussed earlier (Robinson & Duesberg, 1968; Kingsbury, 1970; Webster & Laver, 1971). Skehel (1971) has calculated a mol. wt. of 3.9×10^6 for the genome of influenza virus. The ribonucleoprotein, too, is in pieces which correspond to the pieces of RNA (Pons, 1971). Also in isolated orthomyxovirus nucleocapsids the RNA is not protected against RNase digestion (Kingsbury, 1970). Uukuniemi virus appears to possess a divided genome, too, since extracted RNA sedimented in two peaks at 21 and 27 S, respectively. Its nucleocapsid structure apparently differs from that of toga- and orthomyxoviruses, since the RNA is almost completely protected from the action of RNase; in this respect Uukuniemi virus resembles paramyxoviruses and rhabdoviruses (Pettersson *et al.* 1971). Four pieces of RNA are located in the interior of the lymphocytic choriomeningitis virion, a member of the arenavirus group. Two of these are presumably host-cell RNAs present in ribosomes which may have been engulfed during budding and which have been visualized electron microscopically. For the remaining two virus RNAs an approximate total mol. wt. of 3.2×10^6 has been estimated (Pedersen, 1971). Leukoviruses contain single-stranded RNA with a mol. wt. of about 10×10^6 ; treatment with agents able to break hydrogen bonds between bases has been shown to result in disintegration of the genome into pieces of similar size, mol. wt. about 2.5×10^6 suggesting that the molecule consists of four (or possibly five) subunits (Duesberg, 1970; Vigier, 1970). The RNA is not protected against RNase by structural proteins of the virus after disruption of the envelope with lipid solvents (Duesberg, 1970).

Proteins

Studies with alphaviruses have revealed two main structural proteins associated with the envelope and the nucleocapsid, respectively. In discontinuous polyacrylamide gel systems the presence of a second envelope glycoprotein has been indicated (Schlesinger & Schlesinger, 1972). The highest mol. wt. proteins were identified as being located on the virus surface. After treatment of Sindbis virus with bromelain the virus projections are selectively degraded. This process is accompanied by a loss of glucosamine label from the remaining structure (Compans, 1971). A minimum of three structural polypeptides has been invariably found for flavoviruses. The core protein of alphaviruses has approximately twice the mol. wt. of the corresponding protein of flavoviruses. From the nonarbo togaviruses only RU virus has been analysed; its envelope and capsid polypeptides have mol. wts. close to those of the corresponding alphavirus proteins (Vaheri & Hovi, 1972). Virus-specific protein label has been found accumulating in an additional third electrophoretic zone; when more vigorous conditions of dissociation were employed, a total of eight species of partially resolved polypeptides has been observed (Liebhaber & Gross, 1972). In Table 2 the mol. wts. for the structural polypeptides of togaviruses and of the other virus groups are given. In Uukuniemi virus, also, two proteins were found in association with the envelope and the nucleocapsid, respectively (Pettersson *et al.* 1971). There are at least seven structural polypeptides in the influenza virion; more may be present, depending on the virus strain and cell species studied. The glycoproteins comprise the surface projections which possess the haemagglutinin and neuraminidase activities of the virus. The glycoprotein with a mol. wt.

Table 2. Molecular weights of the structural proteins of togaviruses and other enveloped RNA viruses ($\times 10^{-3}$)

Viruses	References				
Togaviruses alpha flavo rubella		63-51(G)	35-28	18-13	9-8
		65-53(G)			
		63-60(G)	56-45	35-30	Horzinek, 1973
Orthomyxoviruses Influenza A					
	94-81	80-75(G)	70-55(G)	60-53	Kilbourne <i>et al.</i> 1972
Uukuniemi			75-65		Pettersson <i>et al.</i> 1971
Coronaviruses OC 43 (human)					
	210-171(G)				Hierholzer <i>et al.</i> 1972
Leukoviruses Leukemia-sarcoma					
	115-93(G)				
Mouse mammary tumour		100(G)			Nowinski <i>et al.</i> 1972
	90		70	52	Nowinski <i>et al.</i> 1971

(G) indicates glycoproteins

of 75000 to 80000 may be cleaved to two smaller polypeptides with mol. wts. of 50000 to 60000 and 23000 to 30000, respectively, which are held together in the virion by disulphide bonds. The haemagglutinin spike consists of two pairs of these polypeptides (Kilbourne *et al.* 1972). A coronavirus analysed on polyacrylamide gels has been shown to contain a minimum of six structural proteins, four of them containing carbohydrate. When applying the bromelain procedure, two of the glycoproteins could be related to the surface projections (Hierholzer *et al.* 1972). Mammalian leukoviruses were found to contain six major proteins whereas the avian viruses contained seven. Proteins of viruses from different mammalian species had very similar mol. wts., six of them being equivalent to the avian virus proteins. The two largest virus proteins contain carbohydrate and are constituents of the membrane (Nowinski *et al.* 1972). Avian and mammalian leukemia-sarcoma viruses differ from mouse mammary tumour virus, which is also a member of the genus leukovirus (Wildy, 1971), although in protein composition it appears more akin to orthomyxoviruses (Nowinski *et al.* 1971).

Lipids

The structural lipids of an alphavirus (Sindbis) are organized in a bilayer. The polar groups orientated towards the centre of the virion interact directly with the nucleocapsid protein and the outer groups with the peripheral envelope glycoprotein. Protein bridges across the bilayer probably do not exist (Harrison *et al.* 1971). Phospholipids stabilize the virus envelope but are not necessary for virus infectivity (Friedman & Pastan, 1969). The phospholipid and fatty-acid compositions of the virus envelope were distinct from the compositions encountered in whole extracts of the cells in which the virus has been grown, but similar to those of the plasma membranes isolated from infected or uninfected cells (Renkonen *et al.* 1971). Viruses grown in different cell species (chick embryo cells, BHK 21 cells) had similar lipid patterns which has led to the suggestion that the lipid affinities of the envelope glycoprotein(s) may determine their composition (David, 1971).

Spin-label data obtained with influenza virus suggest that myxovirus lipid also is arranged in a bilayer; the glycoprotein projections are probably not involved in the organization of the lipid layer (Landsberger *et al.* 1971). Orthomyxovirus lipids appeared to be entirely of cellular origin, their composition depending on that of the host cell (Webster & Laver, 1971). The situation would thus appear different from that discussed above for togaviruses. However, phospholipid analysis of Venezuelan equine encephalitis virus, an alphavirus, showed that virus propagated in L-cells (mouse origin) had a composition different from chick embryo cell virus, which was also reflected by a different sensitivity to thermal inactivation (Heydrick, Comer & Wachter, 1971). The problem of the specificity in lipid affinities of the envelope proteins has recently been reconsidered by Laine *et al.* (1972). The authors observed that the fatty acids of the different lipid classes present in the virus and host cell plasma membranes are quite similar; furthermore, some of the virus lipids occur in such small quantities that there is fewer than one molecule of them for every envelope protein molecule. When the phospholipids of members of three different groups of viruses (toga-, paramyxo- and leukoviruses) grown in the same host cell species were compared, similar patterns were observed (Quigley, Rifkin & Reich, 1971). All these findings speak against specific lipid affinities in the virus envelope polypeptides.

ANTIGENIC CHARACTERISTICS

Haemagglutinin

Alpha- and flavoviruses agglutinate erythrocytes of a number of animal species in a defined pH range; this simple and rapid technique has been standardized by Casals' group and is used in many laboratories for identification and antigenic grouping of arboviruses. The search for a haemagglutinating activity in nonarbo togaviruses was successful so far only with RU virus (Halonen, Ryan & Stewart, 1967; Stewart *et al.* 1967), whereas all attempts to demonstrate it with the other viruses failed. The presence of surface projections as visualized by electron microscopy in the arthropod-borne togaviruses and RU virus correlates with their haemagglutinating activity. The nonarbo LDH, EA, VD and HC viruses show a smooth surface and are devoid of this property. Since the experiments of Osterrieth (1965*a, b*) and Mussgay & Rott (1964) it is generally agreed that the projections are the morphological substrate for the specific haemagglutinating activity of togaviruses (alphagroup); the same has been proven or suggested for myxoviruses (Laver & Valentine, 1969), coronaviruses (Kaye & Dowdle, 1969), leukoviruses (Schäfer & Szántó, 1969) and members of the Bunyamwera supergroup (Taylor, 1967). Treatment of alphaviruses with proteases, e.g. caseinase, bromelain or pronase resulted in a decrease in haemagglutinating activity of the preparations with concomitant appearance of smooth-surfaced virus particles in the electron microscope. The glycoprotein nature of the alphavirus projections has been identified by Compans (1971). However, the envelope glycoprotein(s) with mol. wts. between 50000 and 60000 in virus completely dissociated by SDS, urea and reducing agents are devoid of haemagglutinating activity, indicating that a certain minimum size or complexity of the structure responsible for this activity is required. When milder conditions have been used to disintegrate alphaviruses components have been released with retention of their haemagglutinating capacity. The size of the fragments differed after various types of treatment, being largest in material treated with Tween 80-ether, intermediate after incubation with sodium deoxycholate and smallest after NP 40 treatment. These differences have been considered as the result of varying degrees of disruption produced by a fundamentally similar process which is based on the removal of lipid essential for the structural integrity of the virus envelope. In gel-filtration experiments a minimum size of the haemagglutinating subunit in excess of 200000 has been indicated (Appleyard, Oram & Stanley, 1970). Parallel haemagglutination and gradient immunodiffusion experiments demonstrated that the HA activity was progressively inactivated with increasing detergent concentration; the non-haemagglutinating envelope fragments still reacted with antibody. Measurements of the angle included between antigen trough and precipitation line led to the estimation of a particle weight of about 180000 daltons for the smallest active split product, suggesting a trimer of the envelope glycoprotein (Horzinek & Mussgay, 1972). The probability of the haemagglutinin being an aggregate of several molecules of envelope glycoprotein in association with lipid has also been discussed by Appleyard *et al.* (1970). When comparing these results with the observations made during characterization of myxovirus haemagglutinin subunits (Laver & Valentine, 1969) it appears logical to ascribe a complex structure to the entity forming erythrocyte-dimers also in togaviruses. In the presence of SDS the subunits of influenza A₁ virus were adsorbed to but did not agglutinate red cells. Thus they appear to be monovalent. When the detergent was removed, the subunits were shown in the electron microscope to aggregate in the form of clusters of radiating rods. Simultaneously they acquired haemagglutinating activity and possessed a wide range of sedimentation coefficients. However, in contrast to myxoviruses it may be assumed that for accomplishing

haemagglutination by togaviruses a specific conformation of the glycoprotein envelope projections is required which is abolished by loss of hydrogen ions and by the presence of defined counterions, as indicated by the limited range of acid pH in which the reaction occurs and its inhibition by Ca^{2+} , but not Mg^{2+} ions (Horzinek & Mussgay, 1972).

Neutralization

A particular behaviour in the neutralization reaction has been described for several togaviruses. Neutralization of EA virus by both immune horse and rabbit sera is enhanced in the presence of unheated guinea-pig serum; the effect was shown to be caused by a sensitizing effect of anti-virus IgG (Hyllseth & Pettersson, 1970). Neutralization of WEE virus by homologous antibody was enhanced about fourfold in the presence of complement, irrespective of whether early or late antibodies were tested (Yoshino, Morishima & Aoki, 1971). Complement-potentiated neutralizing antibodies were also detected in sera of rabbits immunized with a flavovirus (Japanese B encephalitis); it was concluded that they had been induced by virion-associated host-cell components since they were not strictly virus specific but showed dependence on the cell in which the virus had been grown (Iwasaki & Ogura, 1968). Several VD virus strains were neutralized by rabbit antisera to uninfected bovine kidney cells, the strain passage history having no importance; antisera to porcine kidney cells, however, failed to neutralize the virus even when grown in this cell species (Fernelius & Packer, 1969). The effect of unheated antiserum on RU virus has been investigated by Almeida & Laurence (1969), who found partially degraded virions with holes about 10 nm in diameter present in the envelope; these virus particles were mostly penetrated by negative stain. Similar changes have been observed in a coronavirus (avian infectious bronchitis virus) after incubation with unheated immune serum; antiserum to uninfected chick cells was able to produce similar lesions (Berry & Almeida, 1968).

The question arises whether this mechanism of antigen-antibody-complement interaction can really be regarded as virus neutralization. It may rather be a confined destruction of the virus membrane by immune lysis which is followed by penetration of RNase; in togaviruses it could digest the nucleic acid, since the nucleocapsid is penetrable by this enzyme.

A different mechanism seems to be involved in the complement potentiation of neutralization of herpes virus (Yoshino & Taniguchi, 1966). Since maximal enhancement has been found already after the early steps in the complement activation sequence, it was suggested that simply covering the virion surface with complement components increases the amount of neutralized virus (Daniels *et al.* 1970).

The presence of an infectious virus-antibody complex in the blood of mice infected with LDH virus has been evidenced by Notkins *et al.* (1966), who showed that sensitized virus could be neutralized by the action of anti-gammaglobulin (Notkins *et al.* 1968). These authors, too, arrived at the conclusion that more extensive coverage of the virion surface with anti-immunoglobulins than with antiviral antibody alone has caused neutralization (Notkins, 1971a).

Antigenic relationships

Until now no antigenic relationship of RU virus to any other animal virus has been described and no differences between strains could be detected (Banatvala & Best, 1969). When RU antisera and antigens were tested against different reagents of structurally related and unrelated arboviruses, no cross-reaction has been observed in haemagglutination inhibition experiments (Mettler, Petrelli & Casals, 1968). Also for EA and LDH virus serologic relationships to other viruses have not been reported so far. An antigenic relationship

between VD and HC virus has been established by Darbyshire (1960) in gel-diffusion experiments and repeatedly confirmed. Also cross-immunity in calves and pigs could be produced after injection of the heterologous virus (Beckenhauer *et al.* 1961). Among several VD virus strains studied, an antigenic gradation was noted with respect to their relationship to HC virus (Castrucci *et al.* 1971), which is compatible with the existence of antigenic types in VD virus. On the basis of cross-neutralization tests using strain-homologous and heterologous sera, three types have been identified which were also related by the appearance of c.p.e. produced in embryonic bovine kidney cells (Fernelius, Lambert & Booth, 1971).

Serologic cross-reactions between members of the alpha- and flavogroups, respectively, occur most extensively in the haemagglutination inhibition reaction, which forms the basis for the present classification (Casals, 1961). From the data available at present, it would seem that the common antigens which account for the relationships between togaviruses are situated at the virus surface rather than in the interior of the particle.

ULTRASTRUCTURAL ASPECTS OF VIRUS MULTIPLICATION

Two frequently noted morphogenetic characteristics of togaviruses are the intense vacuolization of infected cells and the maturation of virus particles in association with membrane structures, either intracellular or at the marginal cell membrane. Alpha- and flavoviruses appear to differ with respect to their morphogenesis, the former enveloping their nucleocapsids by budding preferentially from the marginal membrane whereas flavoviruses emerge from internal vacuolar membranes. It is expected that the general mode of maturation of alpha- and flavoviruses may be similar; the site of maturation and the accumulation of precursor particles (in alphaviruses) may be responsible for the characteristic differences in appearance of cells infected with viruses of the two groups.

In RU virus-infected cells some budding from intracytoplasmic membranes but mostly from marginal membranes occurs (Murphy, Halonen & Harrison, 1968; Van Bonsdorff & Vaheri, 1969), the general picture resembling that of alphaviruses. In HC virus only very rarely were virions seen to bud from the marginal membrane but the Golgi apparatus seemed to be involved in morphogenesis (Scherrer *et al.* 1970). Envelopment of most nonarbo togaviruses studied so far appears to be very efficient; intracytoplasmic nucleoid accumulation can be detected only in cells infected with LDH virus (du Buy & Johnson, 1966).

It has been shown that during the budding process of an alphavirus a coating of projections is added to the outside of the membrane concomitant with the extrusion of the enveloping nucleocapsid into the extracellular space (Acheson & Tamm, 1967). Recent ferritin antibody studies suggested that either the incorporation of the virus envelope glycoprotein into the cell membrane is under control of the progeny nucleocapsid or the capsid is assembled adjacent to those portions of the membrane which have incorporated virus-determined components (Pedersen & Sagik, 1972). Most influenza virions are released from the surface membrane of the infected cell but occasionally particles appear to be budding into vacuoles. In the latter case, however, it was found that the vacuoles were either continuous with or derived from the cell surface, indicating that orthomyxovirus maturation may take place exclusively at a single type of membrane (Compans & Dimmock, 1969). All members of the Bunyamwera supergroup studied develop by budding into intracytoplasmic cisternae in the region of the Golgi complex, where they may accumulate in masses in membraneous enclosures (Murphy, Harrison & Tzianabos, 1968; Holmes, 1971). Coronaviruses most closely resemble Bunyamwera viruses in mode and site of maturation, accumulation in distended cisternae being an especially striking similarity. They clearly

differ from myxoviruses by the lack of budding from the cellular membranes (Bradburne & Tyrrell, 1971; Cunningham *et al.* 1972). Arenaviruses preferentially emerge from the plasma membrane but occasionally virus particles bud from intracytoplasmic membranes as well, accumulating within vacuoles (Murphy *et al.* 1970). Leukoviruses are released mainly from the plasma membrane but budding into cytoplasmic vacuoles and channels of the endoplasmic reticulum has also been found (Vigier, 1970).

Membrane structures evidently participate in the maturation of all lipid-containing RNA viruses. Envelopment of nucleocapsids to form infectious virions may occur either at the cell surface or along cytoplasmic membranes or at both of them. Accumulation of naked spherical nucleocapsids around vacuoles, however, is characteristic for alphaviruses and is missing in all other infections. Of the togaviruses, these have been most intensely studied and attempts to correlate ultrahistological alterations and synthesis of virus-specified macromolecules have been made. In the cytoplasm of alphavirus-infected cells unique membranous structures could be found consisting of membrane-limited vacuoles which enclose regularly attached membraneous spherules (type I cytopathic vacuoles). Evidence has been presented that these structures are the sites of alphavirus replication, since their number decreased in the presence of inhibitors of virus RNA synthesis (guanidine) or of virus protein synthesis (cycloheximide); high-resolution autoradiography of infected cells showed accumulation of RNA label at the type I vacuole (Grimley, Berezesky & Friedman, 1968; Grimley *et al.* 1972). When cytoplasmic extracts were subjected to isopycnic centrifuging these structures were found accumulating in a fraction (density 1.16 g/ml) which was heavily enriched in pulse-labelled RNA, virus RNA polymerase and virus RNA forms associated with RNA replication. The fraction was considered to contain a membrane-associated replication complex of a type previously defined in picornavirus infection.

The virus protein appeared at a higher density (1.21 g/ml) in fractions which contained rough endoplasmic reticulum and virus nucleocapsids (Friedman *et al.* 1972). Also the virus-specific polypeptides of a flavovirus (Japanese B encephalitis) synthesized during infection were all bound to membranes. None of them could be completely released by salt and detergent treatment alone; incubation with trypsin resulted in a characteristic sequence of release which was interpreted in terms of an orderly association of the larger virus proteins with cytoplasmic membranes (Shapiro *et al.* 1972).

CONCLUDING REMARKS

When comparing the properties of other enveloped RNA viruses with those of members of the togafamily, some unique properties can be attributed to them. They are distinctly smaller than orthomyxo-, Bunyamwera supergroup-, corona-, arena- and leukoviruses as judged from electron micrographs and sedimentation coefficients (see Table 1). Their nucleocapsid is a globular particle whose cubic substructure has been proven for the alphagroup whereas from the other viruses (with the exception of arenaviruses, where no pertinent information is available) elongated structures are released upon pretreatment, displaying helical symmetry. The genome of togaviruses is infectious, which has not been reproducibly shown for members of the other groups. It consists of a single-stranded polyribonucleotide with a mol. wt. of 3 to 4×10^6 which is in one piece; in contrast, indications for subgenomic fragments of RNA, as in the case of orthomyxoviruses, exist for Uukuniemi virus and arenaviruses.

It is understood that this review on the comparative aspects of togaviruses contains, so to speak, average information, which, on one hand, has not been obtained for all members

of the family and, on the other, does not take into account conflicting results. The taxonomic position of LDH virus may still be questioned. Elongated virions have been encountered with a frequency uncommon in other togaviruses (Prosser & Evans, 1967) and a genome mol.wt. of 6 to 8×10^6 has been calculated from radiation target analysis (Rowson, Parr & Alper, 1968); this figure would be about twice the value determined for togaviruses using sedimentation or gel electrophoresis analysis. It must be noted that the RNA data for another lipid-containing single stranded RNA virus (Rous sarcoma virus) as determined by radiation target analysis differ by a factor of 5 from the value obtained by other means (Ginoza, 1967). Inhibition of LDH virus multiplication by actinomycin D (Notkins, 1971*b*) has been observed in contrast to the findings of most workers in the togavirus field; however, the multiplication of a flavovirus (Japanese B encephalitis virus) also has been inhibited by this drug (Zebowitz, Leong & Doughty, 1972).

When taxonomy is discussed the introduction of hierarchical aspects is inevitable. However, the use of categories such as species, genus and family has proved practical; in the togavirus family, the two genera alphavirus and flavovirus are defined by their antigenic interrelatedness and by the difference in the mol. wts. of the nonglycosylated nucleocapsid proteins. For classification of the serologically unrelated nonarbo togaviruses the size of the capsid protein may become an essential criterion. Envelope glycoproteins with mol. wts. of 50000 and more appear to occur in all structural groups (Table 2). Judging from the polypeptide species participating in virion architecture, one is tempted to regard togaviruses and Bunyamwera supergroup viruses as comparatively primitive. However, the number of proteins is not necessarily correlated with the level of structural complexity. As an example, Sindbis and vesicular stomatitis virus, which differ considerably in morphology and structure, both possess genomes of similar size and three main virus polypeptides. Their molar ratio is 1:1:1 in Sindbis (Schlesinger & Schlesinger, 1972) and 1:2:3 in vesicular stomatitis virus, as shown by Cartwright *et al.* (1972). The authors proposed attractive models for this rhabdovirus which take into account the number and proportion of the structural subunits and their possible interactions. It is clear from this comparison that mutual interactions of the virion polypeptides, rather than their size or absolute number, determine its structural complexity. Since these parameters cannot yet be quantitated, it is felt that the anatomy of enveloped viruses must be derived from results of experiments employing stepwise degradation and electron microscopy.

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