A STUDY OF AUSTRALIAN ARBOVIRUSES RESEMBLING BLUETONGUE VIRUS

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Abstract

Five Australian arboviruses, D'Aguilar, Eubenangee, Corriparta, and two as yet unnamed isolates Ch9935 and MRM10434 were studied by thin-section and negative-contrast electron microscopy and were found to be very similar in both morphology and morphogenesis. In thin section they had an extremely electron-dense core surrounded by a less dense capsid. All developed in association with a fibrillogranular intracytoplasmic matrix and to varying degrees were released as either enveloped or unenveloped particles from the infected cell. The diameters of enveloped and non-enveloped particles were 100–104 and 66–67 nm respectively. Negatively stained particles exhibited a surface capsomer structure.

Comparative sodium deoxycholate sensitivity studies were carried out on the five viruses and a known sensitive virus—Semliki Forest virus. Results indicated a relative resistance to the detergent in the case of all of the Australian arboviruses.

Coriphosphine-O staining of VERO cells infected with D'Aguilar, Ch9935, or MRM10434 viruses showed the formation of green and later flame red intracytoplasmic inclusions.

The resemblances between these Australian arboviruses and reovirus, bluetongue virus, and similar overseas isolates are discussed.

I. INTRODUCTION

In recent years a group of arboviruses showing some resistance to lipid solvents and a morphological resemblance to bluetongue virus and the reoviruses has emerged from the arbovirus family. A number of arboviruses fitting into this group have been isolated in Australia—in northern Queensland. These are Corriparta (Doherty *et al.* 1963), Eubenangee (Doherty *et al.* 1968), D'Aguilar (Doherty *et al.* 1969), and two, as yet, unnamed isolates Ch9935 and MRM10434 (Doherty *et al.* 1970). No antigen common to all five of the Australian arboviruses has been found. However, a serological relationship between the two unnamed isolates and relationships between some of the Australian viruses and certain overseas isolates fitting into this morphological group have recently been found (Borden *et al.*, unpublished data).

The initial determination of the resistance of all of these Australian arboviruses to lipid solvents was carried out at the Queensland Institute of Medical Research by

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Dr. R. L. Doherty and co-workers, with more detailed chemical studies reported on Corriparta by Carley and Standfast (1969), and on all five viruses by Gorman, Wham, and Symons (1970). Carley and Standfast (1969) reported the morphology of Corriparta virus and showed that its nucleic acid is probably of the ribose type. No chemical evidence is yet available on the nature of the nucleic acids of the other four arboviruses studied here. A preliminary study of the morphology and morphogenesis of Eubenangee virus has already been published (Schnagl, Holmes, and Doherty 1969).

The present study was undertaken to characterize these arboviruses further, particularly the three more recent isolates. Using thin-section and negative-contrast techniques to demonstrate morphology and morphogenesis, we have compared D'Aguilar, Ch9935, and MRM10434 with Corriparta and Eubenangee and with the published findings on the overseas members of this virus group. In addition, a comparative study on the sodium deoxycholate resistance of all five of the Australian viruses was carried out, as definitive information of this sort has not been published for these viruses. In order to obtain preliminary information on the nature of the nucleic acid in D'Aguilar, Ch9935, and MRM10434 viruses, a direct comparison with reovirus type 3 was made in infected VERO cells stained with coriphosphine-O.

II. Materials and Methods

(*a*) *Viruses*

The arbovirus strains used were kindly supplied by Dr. R. L. Doherty, Queensland Institute of Medical Research, Herston, Qld. These viruses—Eubenangee (strain In1074), Corriparta (strain MRM1), D'Aguilar (strain B8112), Ch9935, and MRM10434 were all obtained as either frozen or freeze-dried pooled suckling mouse brains.

Reovirus type 3 (Abney strain) was obtained as the National Institute of Health research reference reagent and propagated in L cells.

(*b*) *Experimental Animals*

Litters of 1-day-old Melbourne University Albino strain mice were infected by intracerebral inoculation of 0·015 ml of diluted pooled mouse brains for either virus titration or stock preparation. For electron microscopy brain tissue was harvested when animals were moribund.

(*c*) *Cell Cultures*

Monolayers of the VERO line of African green monkey kidney cells were grown in medium 199 containing 5% foetal calf serum. After infection, the monolayers were maintained on the same medium but with 1·5% foetal calf serum.

(*d*) *Electron Microscopy*

Infected mouse brains were randomly cut into approximately 1 mm^3 blocks and fixed in Millonig buffered 4% (v/v) glutaraldehyde for 1½–2 hr at 4°C. After post-fixation in Millonig-buffered 1% (w/v) osmium tetroxide for 30 min at 4°C the specimens were dehydrated in acetone and embedded in Araldite. Infected cells, after harvesting by scraping off the glass, were pelleted by low-speed centrifugation and the pellets fixed and embedded as were the mouse brain specimens. Sections were cut with glass knives on a Huxley ultramicrotome and stained with uranyl acetate and then lead citrate.

For examination of virus by negative staining infected mouse brain material was homogenized in pH 9·0 borate saline and the homogenate clarified firstly by centrifugation at 1000 *g* and than at 10,000 *g* for 10 min. The viruses were then pelleted by spinning at 80,000 *g* for 1 hr and the pellets resuspended in pH 9·0 borate saline. Cell-culture-grown virus was similarly partially purified after infected cells had been disrupted by ultrasonic vibration for 1 min at 20 kHz on a 60 W MSE ultrasonic generator in the supernatant medium. The resuspended virus was stained with 2% potassium phosphotungstate using the "loop drop" method or a pseudo-replication technique (Jamison and Mayor 1966).

Micrographs were taken using a Hitachi HU-IIA electron microscope operating at 50 kV. Magnifications were calibrated using catalase crystals (Luftig 1967).

(*e*) *Sodium Deoxycholate Sensitivity Tests*

Suspensions of 10% (w/v) virus-infected suckling mouse brain in borate saline (pH 9·0) containing 0·75% bovine serum albumin were clarified by centrifugation at 10,000 r.p.m. for 1 hr in a rotor No. 40, of a Spinco preparative ultracentrifuge. The supernatants were then exposed to final sodium deoxycholate concentrations of either 0·1% or 0·2% for 1 hr at 37°C (following the method of Sunaga, Taylor, and Henderson (1967). Controls were exposed to the bovine serum albumin buffer without added sodium deoxycholate. Tenfold dilutions of the residual virus were prepared in bovine serum albumin buffer and titrated by intracerebral inoculation into 1-day-old suckling mice.

Titres were calculated according to the method of Reed and Muench (1938). Semliki Forest virus, representing the togaviruses, was included as a sodium deoxycholate-sensitive control.

(*f*) *Coriphosphine-O Staining*

VERO cells were grown on coverslips in Petri dishes and infected with the appropriate virus when confluent. Coverslips were then removed for staining at various intervals.

The method of staining with coriphosphine-O was a modification of that outlined by Keeble and Jay (1962).

Coverslips were rinsed in phosphate-buffered saline at pH 7·0 and then fixed in Carnoy's fluid for 10 min. After a rinse in phosphate-buffered saline containing 0·2% phenol, the coverslips were stained for 5 min in a 0·005% coriphosphine-O solution which was prepared by diluting a stock 0·05% coriphosphine-O solution in 0·2M acetate buffer, pH 4·4, 1 in 10 in phosphate-buffered saline plus 0·2% phenol. The coverslips were then rinsed thoroughly for 10 min in the above diluent and mounted in this medium on glass slides.

Specimens were observed with a Leitz Wetzlar fluorescence microscope with an automatic camera attachment under ultraviolet bright field. Black and white prints were prepared from colour slides taken on Agfa CT18 film.

III. Results

(*a*) *Thin-section Electron Microscopy*

Since all five viruses appeared morphologically very similar, they can be discussed essentially as a group.

In thin sections of infected mouse neurons and infected VERO cells, virions characteristically appeared within and around discrete fibrillogranular intracytoplasmic inclusions. These inclusions were fairly irregular in shape and unbounded (Fig. 1). The virus particles themselves were approximately spherical and possessed an extremely electron-dense core surrounded by a more electron-lucent layer interpreted as capsid (Figs. 1 and 2). Average diameters for D'Aguilar, Ch9935, and MRM10434 particles were very similar: 66 ± 4, 67 ± 4, and 67 ± 4 nm, respectively. Corresponding core diameters were 36 ± 3, 38 ± 3, and 38 ± 3 nm. These dimensions are similar to those

already reported for Eubenangee (Schnagl, Holmes, and Doherty 1969) and Corriparta viruses (Carley and Standfast 1969).

In the case of D'Aguilar virus infection of VERO cells, very large numbers of tubular structures in the size range of spindle tubules (microtubules) were frequently observed in close proximity to virus particles. These tubules appeared far more frequently in the virus-infected cells than in uninoculated control cells. Similar tubules were noted in MRM10434- and Ch9935-infected VERO cells but somewhat less frequently than in cells infected with D'Aguilar virus.

Occasionally a different, wider type of filamentous structure, of variable diameter and length, was seen in the cytoplasm of D'Aguilar-infected VERO cells. The virus seemed to be more intimately associated with these structures (Fig. 3). These filaments were clearly distinguishable from the previously mentioned tubules although they could possibly be interpreted as "coated" tubules (Dales 1963).

In D'Aguilar-infected VERO cells, intranuclear filaments with diameters and morphology somewhat similar to those of the dense intracytoplasmic filamentous structures were occasionally seen (Fig. 4). Some evidence of cross-striation was apparent on both the intranuclear and intracytoplasmic dense filaments. Neither of these latter types of filamentous structures was seen in cells infected with any of the other four viruses.

Release of virus from the infected cell occurred at least partly by a budding process at the cell margin in the case of all five viruses (Figs. 5 and 6). The resultant extracellular-enveloped particles (Fig. 7) had diameters of 100 ± 5 (D'Aguilar), 103 ± 5 (Ch9935), 104 ± 5 (MRM10434), and 100 ± 4 nm (Corriparta)—cf. 101 ± 4 nm for Eubenangee (Schnagl, Holmes, and Doherty 1969). However, cell dissolution accompanied by release of unenveloped particles was also frequently observed. This latter method of virus release seemed to be predominant in the case of D'Aguilar virus and was definitely common in the cases of Corriparta, Ch9935, and MRM10434. Budding, however, seemed to predominate in the case of Eubenangee virus.

In cells infected with D'Aguilar virus intracytoplasmic accumulation of virions, occasionally forming paracrystalline arrays, occurred late in the growth cycle. This was not apparent with the other four viruses.

(*b*) *Negative-contrast Electron Microscopy*

Particles of D'Aguilar, Ch9935, and MRM10434 viruses were observed in negatively stained preparations from both infected mouse brain and infected cell cultures. They were all similar, appearing polygonal with an obvious capsomer structure and diameters of 70 ± 3, 71 ± 4, and 71 ± 3 nm respectively (Fig. 8). These mean diameters are somewhat larger than those reported for Corriparta (Carley and Standfast 1969) and Eubenangee (Schnagl, Holmes, and Doherty 1969), although morphologically the particles of these latter two viruses appeared similar to those of D'Aguilar, Ch9935, and MRM10434.

Particles with a closely fitting envelope and dimensions in accord with those obtained for enveloped particles in thin section were not definitely identified in any of the preparations studied. Occasionally, particles were surrounded by what appeared to be large and very loosely fitting membranes ("pseudoenvelopes"). Such membranes occasionally surrounded more than one virus particle.

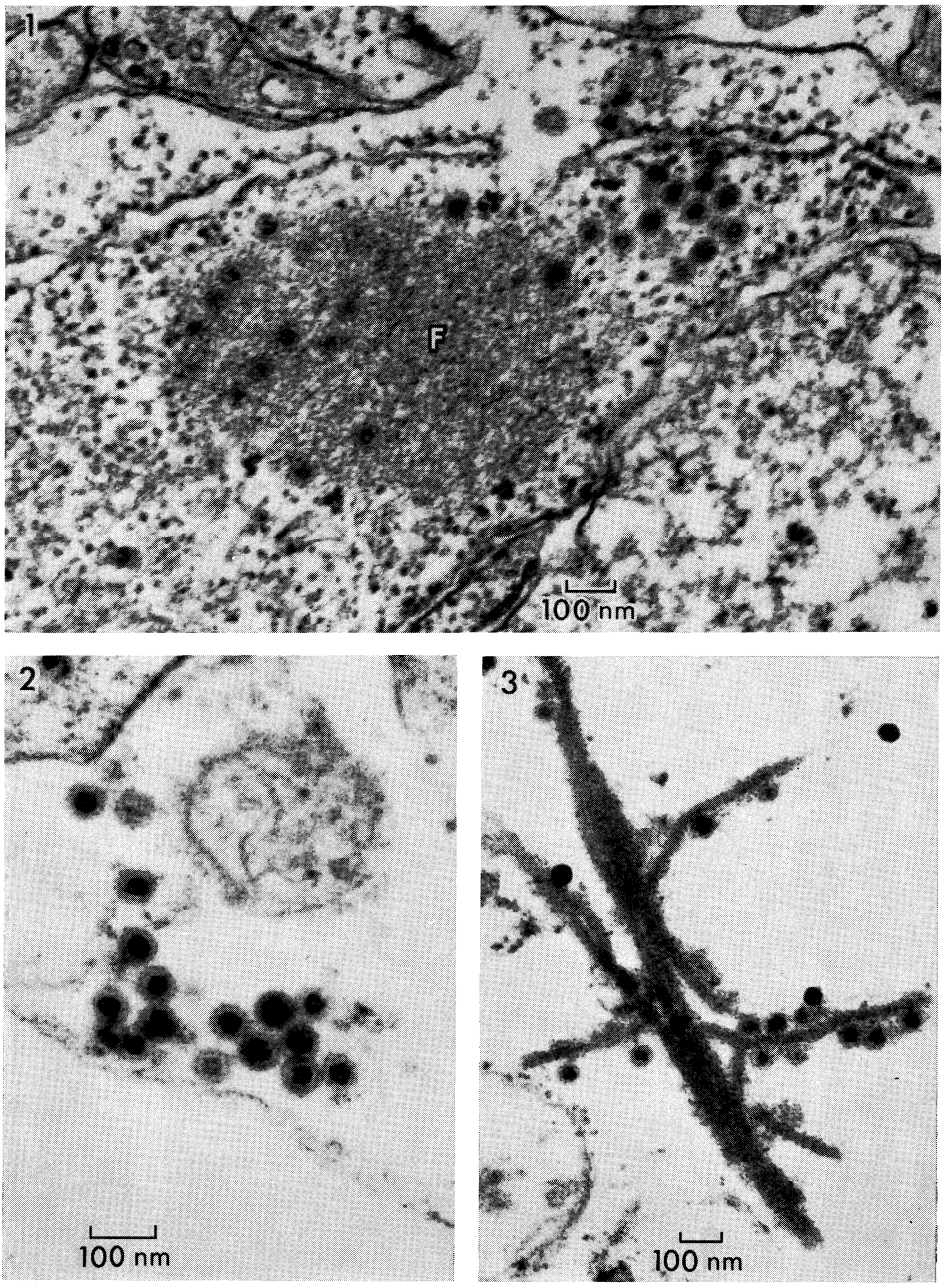
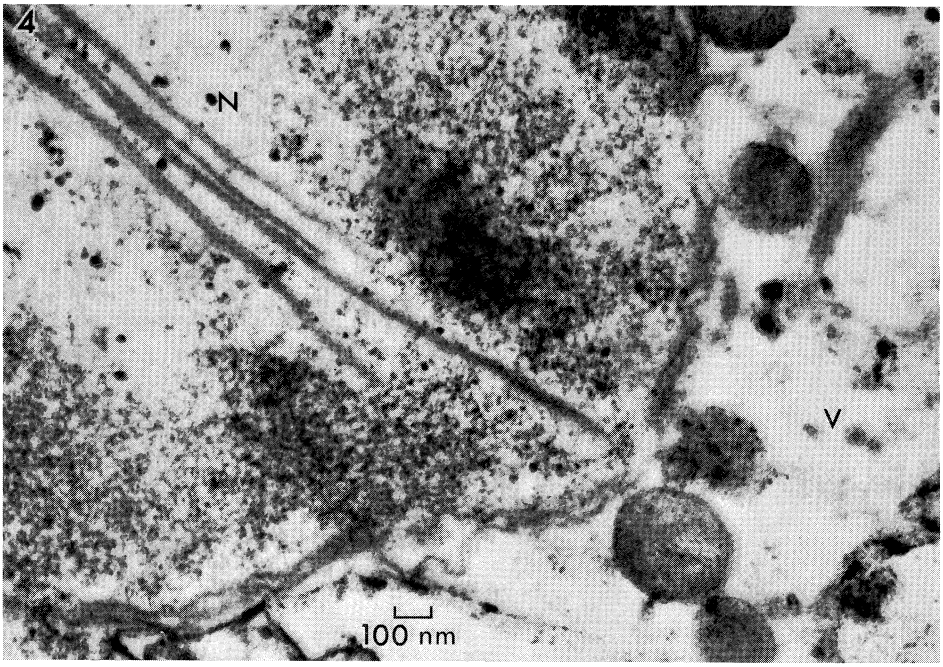


Fig. 1.—Cytoplasmic focus (*F*) of D'Aguilar virus infection in mouse brain. ×64,000.

Fig. 2.—Particles of D'Aguilar virus free in the cytoplasm of a VERO cell. ×90,000.

Fig. 3.—Dense intracytoplasmic filaments in a VERO cell infected with D'Aguilar virus. Note virus particles apparently attached to the filaments. ×58,000.



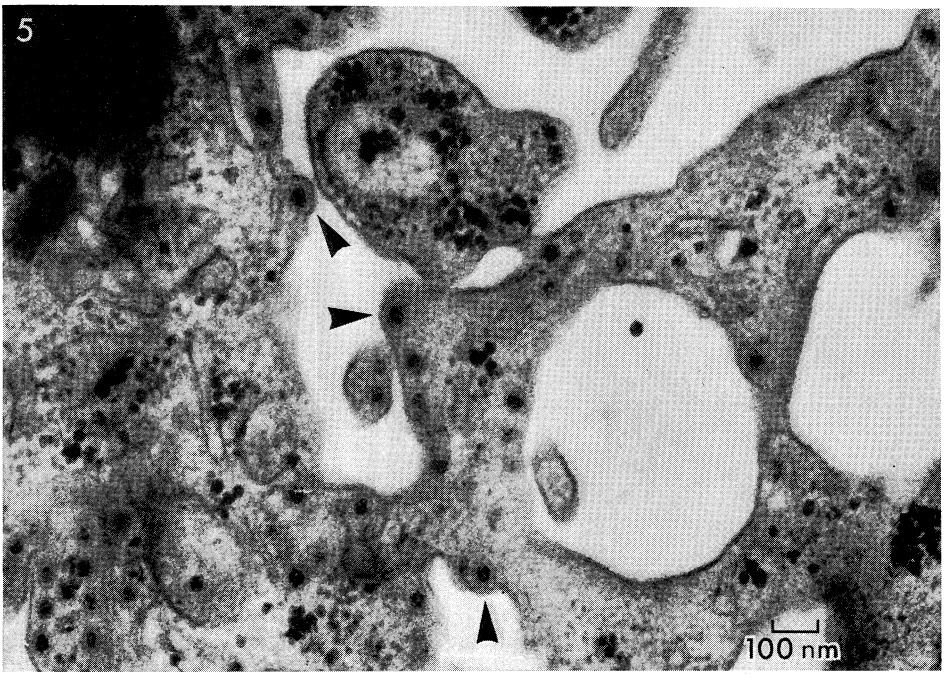
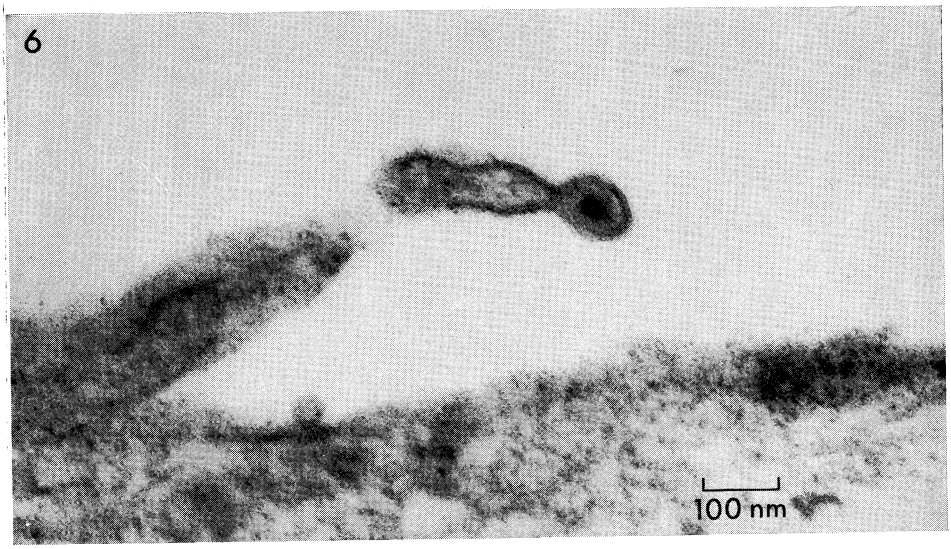
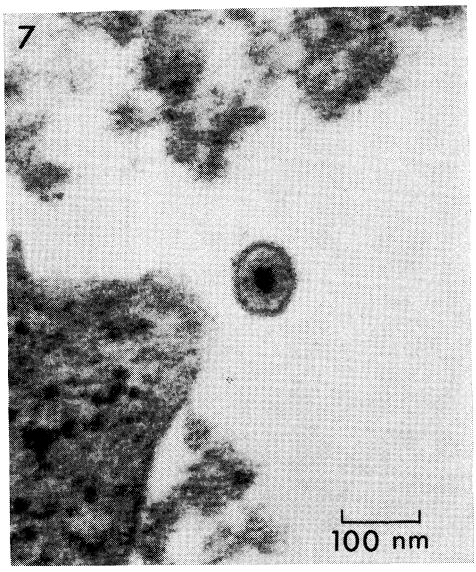


Fig. 4.—Intranuclear filaments (*N*) in a VERO cell infected with D'Aguilar virus. Note virus particle in cytoplasm (*V*). ×50,000.

Fig. 5.—Corriparta virus budding from VERO cell margins (arrows). ×60,000.





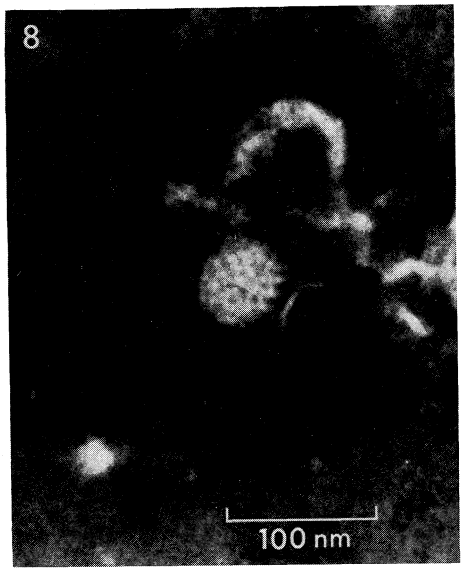


Fig. 6.—MRM10434 virus about to be budded off from a cytoplasmic process of a VERO cell. ×100,000.

Fig. 7.—Budding or extracellular-enveloped virion of MRM10434 virus from a VERO cell. ×100,000.

Fig. 8.—Negatively stained virus particle in mouse brain preparation of Ch9935 virus. Note capsomer structure. ×200,000.

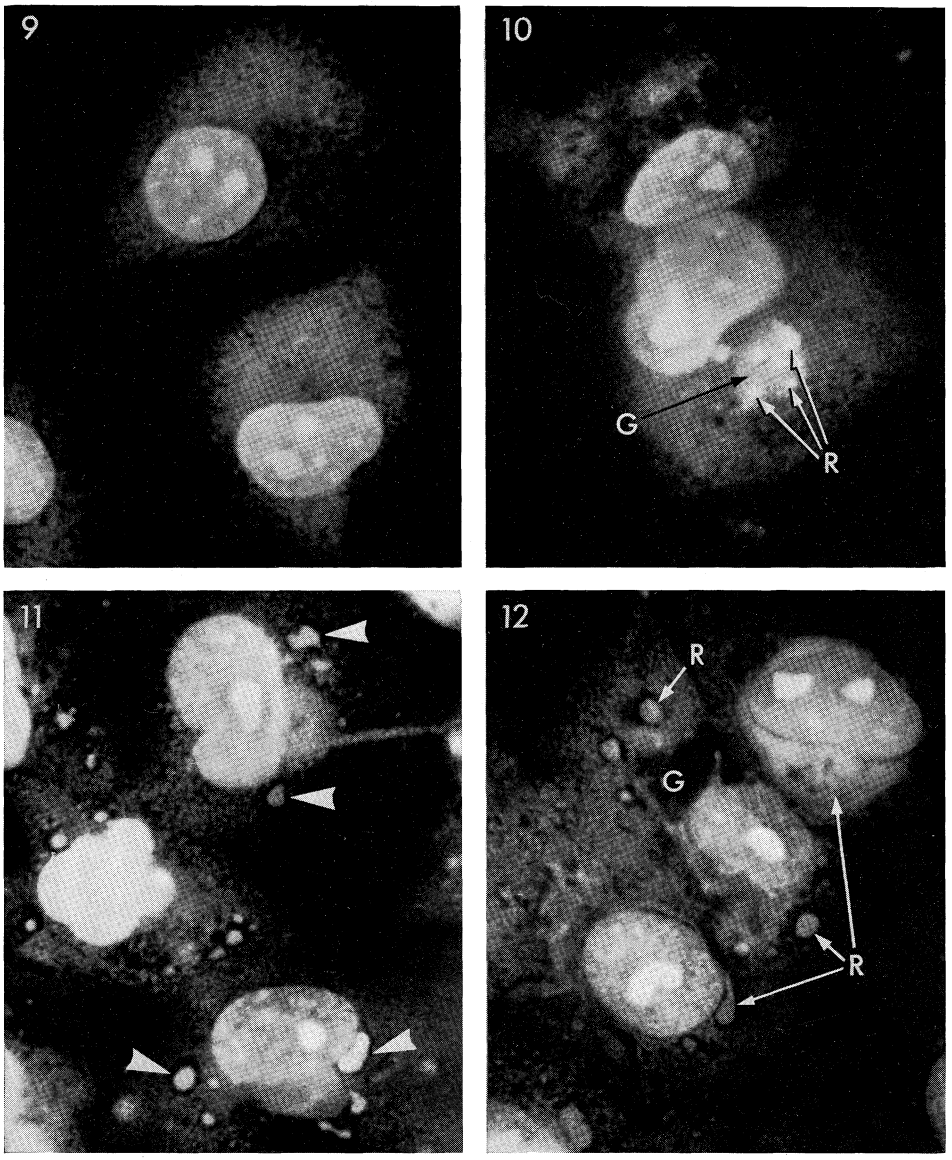


Fig. 9.—Normal VERO cells stained by the coriphosphine-O technique. [Figures 9–12 are black and white copies of colour originals and are all ×640.]

Fig. 10.—Small early red inclusions (*R*) within a more extensive intracytoplasmic green inclusion (*G*). VERO cell infected with Ch9935 virus. Coriphosphine-O staining.

Fig. 11.—Late red inclusions (arrows) in cytoplasm of VERO cells infected with Ch9935 virus. Coriphosphine-O staining.

Fig. 12.—Green (*G*) and yellowish red (*R*) inclusions in the cytoplasm of VERO cells infected with reovirus type 3. Note both isolated and perinuclear arrangements of the inclusions. Coriphosphine-O staining.

(*c*) *Sensitivity of Viruses to Sodium Deoxycholate*

The results for the sensitivity of D'Aguilar, Eubenangee, Corriparta, Ch9935, MRM10434, and Semliki Forest viruses to sodium deoxycholate are summarized in the following tabulation [all titres are in $\log_{10}$ (suckling mouse LD_{50}) per 0·015 ml inoculum]:

Virus	Control Titre	Change in Titre Relative to Control for Sodium Deoxycholate Concentrations of:	
		0·1% (1:1000)	0·2% (1:500)
Semliki Forest	9·7	−1·9	−7·5
Eubenangee	6·1	−1·6	−1·6
CH9935	4·8	−0·6	−1·2
D'Aguilar	5·3	+0·3	−1·2
MRM10434	5·6	−0·4	−1·0
Corriparta	6·7	−0·2	−0·1

Thus, after exposure of virus to a final concentration of sodium deoxycholate of 0·1% (1:1000) the results obtained were equivocal. D'Aguilar and Corriparta showed little change in titre, Ch9935 and possibly MRM10434 showed some sensitivity, while Eubenangee and Semliki Forest viruses showed marked sensitivity. After exposure of virus to a final concentration of sodium deoxycholate of 0·2% (1:500), all of the viruses except Corriparta showed a definite sensitivity although the togavirus (Semliki Forest virus) was inactivated to a much greater degree than the others.

(*d*) *Coriphosphine-O Staining*

Stained uninfected VERO cells (Fig. 9) showed a nucleus with a green fluorescence, occasionally flecked with yellow. This is indicative of the double-stranded nature of the nucleic acid present (DNA). The nucleoli were greenish yellow to red. The cytoplasm fluoresced flame red, indicating the predominantly single-stranded nucleic acid present (RNA).

Observations on Ch9935 will be illustrated here but the results were entirely similar for D'Aguilar and MRM10434 viruses.

The growth of each virus was characterized by the appearance, at an early stage in its cycle, of greenish intracytoplasmic inclusions often adjacent to the nucleus. These were fairly difficult to find, probably because of the relatively low multiplicity of infecting virus. Somewhat later, small points of red fluorescence appeared within or at the edges of these green inclusions (Fig. 10). At this stage, some cells contained numerous small bright red inclusions without any sign of green inclusions. The bright red inclusions became larger and more numerous with time. Frequently, though not invariably, they were in a juxtanuclear position (Fig. 11). The red inclusions were more discrete and sharply defined than the green ones.

In the case of VERO cells infected with reovirus type 3, very similar observations were made. Early in the growth cycle, apple green intracytoplasmic inclusions were evident, followed later by reddish yellow inclusions as well. These reddish yellow inclusions became more numerous with time and often very extensive. The inclusions often showed the "typical" reovirus perinuclear arrangement, but 75% of cells

contained, either in addition or instead, scattered discrete inclusions very like those produced by the arboviruses (Fig. 12).

The intracytoplasmic location and nature of the red inclusions—smaller and discrete in the case of the arboviruses and both small discrete and extensive perinuclear aggregates in the case of reovirus type 3—correlate exactly with observations of the fibrillogranular inclusions in VERO cells made by means of thin-section electron microscopy.

IV. Discussion

The similarities in morphology and morphogenesis between the five Australian arboviruses—D'Aguilar, Eubenangee, Corriparta, Ch9935, and MRM10434—are clearly sufficient to justify their inclusion in a group, despite their lack of any known common antigen. On morphological grounds, and also on the basis of their limited deoxycholate resistance, they can be placed with bluetongue virus (Studdert 1965; Ritchie and Bowne 1967; Bowne and Ritchie 1970; Verwoerd 1970), African horse-sickness virus (Lecatsas and Erasmus 1967; Breese, Ozawa, and Dardiri 1969), and numerous other (currently 19) overseas isolates of the "solvent-resistant arbovirus" group (Borden *et al.*, unpublished data).

Although all these viruses develop in similar intracytoplasmic granular inclusions, several varieties of associated fibrillar structures have been noted. Intranuclear filaments like those in cells infected with D'Aguilar virus have been reported for Colorado tick fever virus only (Murphy *et al.* 1968; Oshiro and Emmons 1968). The similar (dense, cross-striated) cytoplasmic filaments seen in cells infected with D'Aguilar, Colorado tick fever (Murphy *et al.* 1968; Oshiro and Emmons 1968), and possibly African horse-sickness viruses (Breese, Ozawa, and Dardiri 1969) may be of the same type. Whether these are related to the larger paracrystalline inclusions observed both in the nucleus and cytoplasm of cells infected with African horse-sickness virus by Breese and Ozawa (1969) is not clear at present. It is also unknown whether there is a connection between the large numbers of microtubules observed, particularly in cells infected with D'Aguilar virus, and the virus growth cycle. As in the case of reoviruses, the association may only reflect the intracellular sites favoured for development of viral inclusions (Dales 1963).

From thin-section electron microscopic evidence it is apparent that all five of the Australian arboviruses may exist outside the cell in the form of either enveloped or unenveloped particles. Certainly virus release by cell dissolution or some other method resulting in the release of unenveloped particles appears to predominate in the cases of Corriparta, D'Aguilar, Ch9935, and MRM10434, but budding at the cell margin was by no means a rare event. With Eubenangee the release of enveloped particles would seem to be predominant. African horse-sickness virus (Lecatsas and Erasmus 1967), bluetongue virus (Bowne and Ritchie 1970), and Colorado tick fever virus (Murphy *et al.* 1968) have been shown to have enveloped forms. It has been reported, however, that with the overseas isolates of arboviruses resembling bluetongue virus, enveloped forms are rarely seen (Tsai and Karstad 1970; Borden *et al.*, unpublished data).

No fully enveloped particles of the Australian viruses were definitely identified by negative staining. They may not have been recognized as such in our only partially purified preparations—especially if the envelope shows no distinct surface projections.

The envelope may be readily lost even during partial purification. Particles surrounded by a "pseudo-envelope" were similar to those observed for bluetongue virus (Els and Verwoerd 1969) and African horse-sickness virus (Oellermann, Els, and Erasmus 1970).

Although it was obvious that naked capsids of D'Aguilar, Ch9935, and MRM10434 possessed a definite capsomer structure, the capsomers were never seen clearly enough for their arrangement to be conclusively defined.

The results obtained for the sodium deoxycholate sensitivity of D'Aguilar, Eubenangee, Corriparta, Ch9935, and MRM10434 viruses indicate a level of resistance definitely higher than that of the togaviruses. In our case a direct comparison was made between these five viruses and Semliki Forest virus because the methods of carrying out the sodium deoxycholate sensitivity test vary in detail between laboratories. This means that a comparison of the actual values obtained in different laboratories often cannot be made. It is obvious from the table that on a comparative basis the five Australian arboviruses differ in their sodium deoxycholate resistance and this is in agreement with the initial results obtained by Dr. R. L. Doherty at the Queensland Institute of Medical Research. Eubenangee has proved to be the most sensitive of the five Australian viruses. However, the unchanged sensitivity of Eubenangee at the two different sodium deoxycholate concentrations, when compared to the other arboviruses tested, shows the importance of the actual concentration when quoting the degree of sodium deoxycholate resistance of a virus. That Corriparta was shown to be completely resistant to sodium deoxycholate at the two concentrations used is entirely in agreement with the results obtained by Carley and Standfast (1969).

In the experiments on coriphosphine-O staining, the type of inclusions observed and their sequence of development was similar both in cells infected with D'Aguilar, Ch9935, and MRM10434 viruses and in those infected with reovirus type 3. These reovirus observations are in accord with those of Rhim, Jordan, and Mayor (1962) on reovirus type 1 in monkey kidney cells. By acridine orange staining, both green and red inclusions were reported in cells infected with Colorado tick fever virus (Green 1969) but only red inclusions have been reported for bluetongue virus (Livingstone and Moore 1962) or African horse-sickness virus (Ozawa 1967). When taken in conjunction with thin-section electron microscopic evidence, the coriphosphine-O staining results therefore underline the close similarity in morphogenesis between the three Australian arboviruses and reovirus.

Although coriphosphine-O staining (or acridine orange staining) certainly does not in itself provide conclusive evidence, the results obtained also suggest that these arboviruses, like reovirus, may contain double-stranded nucleic acid. Bluetongue virus (Verwoerd 1969; Verwoerd, Louw, and Oellermann 1970), African horse-sickness virus (Oellermann, Els, and Erasmus 1970), and Colorado tick fever virus (Green 1970) have already been shown to possess double-stranded RNA.

From the results presented in this paper we can therefore add several Australian arboviruses to the now sizeable group containing bluetongue virus and reovirus-like arboviruses.

V. Acknowledgments

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