A New Group of RNA Viruses from Insects

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SUMMARY

The isolation and properties of three new insect viruses are described. Two were isolated from the limacodids, Darna trima and Thosea asigna, and one from the saturniid, Philosamia cynthia ricini. The three viruses are closely related to Nudaurelia capensis β virus and the virions of the four are serologically related to, but distinguishable from, each other. Virions of members of the group have a diam. of 35 nm, a sedimentation coefficient of 194 to 210S and a buoyant density in CsCl of 1.27 to 1.30 g/ml. The virion contains between 10 and 11% RNA of mol. wt. about $1.8 \times 10^6$, and 240 molecules of a single polypeptide species with a mol. wt. of 61000 to 63000. The mol. wt. of the virion is about $16 \times 10^6$. These four viruses superficially resemble the caliciviruses of mammals but there are some major differences between the two groups.

INTRODUCTION

Increasing numbers of small RNA viruses isolated from insects have been studied in recent years and, of these, Nudaurelia capensis β virus (N/ßV) possesses several unique properties. The virus is one of five which have been found in the pine emperor moth Nudaurelia cytherea capensis (Lepidoptera: Saturniidae) in South Africa (Juckes, 1970). N/ßV was shown by Juckes et al. (1973) to be serologically indistinguishable from a virus isolated by Grace & Mercer (1965) from the gum emperor moth, Antheraea eucalypti in Australia. The particles of N/ßV have a diam. of 35 nm and contain between 11 and 12% single-stranded RNA with a mol. wt. of about $1.8 \times 10^6$ (Struthers & Hendry, 1974). Polson et al. (1970) estimated the particle weight of N/ßV to be $16.3 \times 10^6$ using a molluscan haemocyanin as a standard. The virus capsid was shown by Struthers & Hendry (1974) to contain a single species of polypeptide with a mol. wt. between 60000 and 62000, and Finch et al. (1974), by combining chemical data with an analysis of electron micrograph images, concluded that the capsid contained 240 copies of the polypeptide, located on a $T = 4$ icosahedral surface lattice, thus distinguishing N/ßV from other similar viruses, whose particles have been shown to be icosahedra with a $T = 3$ lattice.

In this paper, some of the properties of three new viruses are described and these three, together with N/ßV, are shown to form a distinct group based on serological tests, although there are differences in some of their physico-chemical properties.

Two of the viruses were isolated from species of Lepidoptera: Limacodiidae. Darna (Orthocraspida) trima Moore is distributed throughout South East Asia where it is a pest of several crops, including coconut and oil palm. Large populations of this insect can produce almost complete defoliation (Wood, 1968). The second Limacodiid, Thosea asigna Moore,

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tho not as common as \textit{D. trima}, has also been recorded as a pest of oil palm in various parts of Malaysia. The third virus studied is of uncertain origin because it was isolated from diseased laboratory cultures of the Saturniid, \textit{Philosamia cynthia}×\textit{ricini} (a hybrid maintained in the U.I.V. laboratories, Oxford, for several years). However, a virus indistinguishable from the Oxford \textit{Philosamia} isolate has been isolated from stocks of the Eri silkworm, \textit{Philosamia ricini} from New Guinea.

\textbf{METHODS}

\textit{Viruses}. Dead infected larvae of \textit{D. trima} were collected from oil palm plants in Sarawak and Sabah, and of \textit{T. asigna} from Sarawak. Infected larvae of \textit{P. cynthia}×\textit{ricini} were obtained from insect cultures from the Oxford University Field Station, Wytham, and infected larvae of \textit{N. cytherea} capensis from Knysa, Cape Province, South Africa.

\textit{Isolation and purification of virus}. Frozen infected larvae were thawed and extracted in 0.05 M-sodium phosphate buffer (PB), pH 7.2, containing 0.2\% 2-mercaptoethanol (2-ME), by disintegrating them in a Colworth Stomacher 80. The extract was squeezed through cheesecloth and the filtrate shaken thoroughly with an equal vol. of n-butanol/chloroform (1:1 v/v). The emulsion was broken by centrifuging at 19,000 g for 15 min and the aqueous layer was withdrawn. Virus was further purified and concentrated by two cycles of alternate high- and low-speed centrifugation (53,000 g for 90 min, 8000 g for 15 min, 107,000 g for 60 min, 8000 g for 15 min). The pellet was allowed to resuspend in 0.02 M-PB at 9°C after each high-speed centrifugation. The partially pure suspension was then layered on a 10 to 30 \% (w/v) sucrose gradient in 0.02 M-PB and centrifuged at 65,000 g for 90 min. Virus-containing zones were collected through a hole made in the bottom of the tube, diluted with approx. 4 vol. of 0.02 M-PB and centrifuged at 65,000 g for 90 min. Finally, the virus suspension was layered on 6 ml of 36 \% (w/w) CsCl in 0.02 M-PB and centrifuged at 180,000 g for 12 to 16 h at 20°C. The virus band was harvested by piercing the bottom of the tube and displacing the gradient with paraffin oil. CsCl was removed by dialysis against several changes of 0.02 M-PB.

\textit{Determination of sedimentation coefficients}. Sedimentation coefficients were determined in a Beckman Model E analytical ultracentrifuge, fitted with both schlieren and u.v. absorption optics. Virus suspensions (0.5 mg/ml) in 0.1 M-PB, pH 7.2, were centrifuged at 20,410 rev/min with the temperature controlled at 20°C.

\textit{Determination of buoyant density of virus particles}. Buoyant densities of the various virus particles were determined in CsCl gradients. Virus samples were layered on 36 \% (w/w) CsCl and centrifuged in a 6×16.5 ml swing-out rotor at 180,000 g for 12 to 16 h at 20°C. The gradients were sampled by puncturing a hole in the bottom of the tube and collecting 0.2 ml fractions. Selected samples were used to determine density whilst the remainder were diluted fourfold and their absorbance measured at 260 nm.

\textit{Determination of the chemical composition of the virus}. Protein concentrations were determined by the method of Lowry \textit{et al.} (1951) as modified by Eggstein & Kreutz, described by Shatkin (1969). RNA concentrations were estimated using the method of Mejbaum described by Shatkin (1969) using yeast RNA (Sigma grade XI) as a standard. Qualitative tests for the presence of DNA were done by the method of Burton (1956).

\textit{Electron microscopy}. Virus samples in 0.02 M-PB were mixed with an equal vol. of 2 \% (w/v) potassium phosphotungstate, pH 7.0, and examined in an AEI EM6B electron microscope at an accelerating voltage of 60 kV.

\textit{Relative electrophoretic mobility of virus particles}. Purified virus suspensions were electro-
phoresed in a slab gel prepared from 1% (w/v) ‘Seaplaque’ agarose in 0.05 M-PB, which was also the electrode buffer. A current of 150 V was applied for 3 h, at the end of which the virus was rapidly visualized by immersing the gels in 17% (w/v) sodium sulphate. Relative mobility was estimated by reference to the distance migrated by the fastest-moving virus which was taken as unity.

**Electrophoresis of virus polypeptides in SDS-polyacrylamide gels (PAGE).** Samples of the virus particles were solubilized by boiling for 2 min in the presence of 1% SDS, 1% 2-ME, 8 M-urea, 10% (w/v) sucrose and 0.1 M-PB, pH 7.8. Samples (5 to 100 µl) were loaded on to 70 × 5 mm polyacrylamide gels containing 0.1 M-PB, pH 7.8, 8 M-urea, 2% SDS, 0.06% TEMED and 0.05% ammonium persulphate. The acrylamide:methylene bis-acrylamide ratio was 20:1. Electrophoresis was carried out at 2 mA/gel for 30 min, followed by 6 mA/gel for up to 6 h in 0.1 M-PB, pH 7.8, 0.1% SDS and 0.1% 2-ME. Gels were stained overnight in 0.1% Coomassie brilliant blue in acetic acid/methanol/water (10:50:40 by vol.) and destained in several changes of the same solvent. The destained gels were allowed to expand in 7% acetic acid before being scanned in a Pye Unicam SP1800 spectrophotometer linked to a Unicam AR25 linear recorder. Estimations of the mol. wt. of the virus polypeptides were made with reference to the mobility of the following proteins of known size: transferrin, bovine serum albumin, ovalbumin, lactate dehydrogenase, carbonic anhydrase, α-chymotrypsin, myoglobin and cytochrome c.

**Antisera and serological tests.** Antisera to purified virus particles, except *D. trima* virus, were prepared in rabbits by intramuscular injection of 1 ml of antigen (500 µg/ml) on three occasions at weekly intervals. Freund’s complete adjuvant was included in the first injection and incomplete adjuvant in the subsequent injections. The rabbits were bled from an ear vein at weekly intervals and the separated sera were stored at −20 °C.

The original antiserum to a *D. trima* virus preparation had been produced by intraperitoneal injection of 1 ml of antigen (concentration 500 µg/ml) into guinea pigs. The antiserum so produced proved to contain antibodies to two different viruses, but antiserum specific for *D. trima* virus was obtained by absorption with a purified preparation of the contaminating virus.

Gel immunodiffusion tests were done in 1% agarose in phosphate buffered saline in 60 mm Petri dishes with 0.1% sodium azide added as a preservative. The bands of precipitate were allowed to develop at 30 °C for at least 48 h, after which the gels were removed from the dish, washed repeatedly in 0.85% saline, rinsed in distilled water and dried under filter paper on glass plates. The dried gels were stained in 1% naphthalene black in 7% acetic acid followed by destaining in the same solvent until the lines of precipitate were well contrasted. The gels were then rinsed in distilled water before drying.

Complement fixation titres were determined using the procedures described by Busby *et al.* (1964). ‘Chessboard’ type titration plates were employed using dilutions of both antigen and antiserum to determine the optimum antigen concentration and antiseraum titres. Complement was allowed to fix at 4 °C overnight and the plates were scored 6 h after the addition of sensitized sheep erythrocytes. Approximately 50% lysis of the erythrocytes in a well was taken as the end point, providing that the preceding well showed little or no lysis.
Fig. 1. Purified suspension of Darna virus particles stained with 2% neutral potassium phosphotungstate. Some particles show evidence of partial breakdown.

Table 1. Physico-chemical properties of the four insect viruses*

<table>
<thead>
<tr>
<th></th>
<th>DV</th>
<th>NβV</th>
<th>PV</th>
<th>TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Sedimentation coefficient ( (s_{20, w}) )</td>
<td>199</td>
<td>210†</td>
<td>206</td>
<td>194</td>
</tr>
<tr>
<td>Density in CsCl (g/ml)</td>
<td>1.289</td>
<td>1.298</td>
<td>1.275</td>
<td>1.275</td>
</tr>
<tr>
<td>% RNA</td>
<td>11.5</td>
<td>11.5</td>
<td>9.5</td>
<td>10.2</td>
</tr>
<tr>
<td>( E_{260/280} )</td>
<td>1.44</td>
<td>1.45</td>
<td>1.36</td>
<td>1.32</td>
</tr>
<tr>
<td>Relative mobility</td>
<td>0.81</td>
<td>1.00</td>
<td>0.61</td>
<td>0.44</td>
</tr>
<tr>
<td>Mol. wt. of polypeptide ( \times 10^{6} )</td>
<td>62,100 (±700)$§$</td>
<td>62,600 (±1000)</td>
<td>62,400 (±500)</td>
<td>60,800 (±1100)</td>
</tr>
<tr>
<td>Mol. wt. of RNA ( \times 10^{-6} )</td>
<td>ND$</td>
<td></td>
<td>$</td>
<td>1.8‡</td>
</tr>
</tbody>
</table>

* DV = Darna virus, NβV = Nudaurelia β virus, PV = Philosamia virus, TV = Thosea virus.
† Struthers & Hendry (1974).
‡ Standard error of the mean.
$||$ ND = not determined.

RESULTS

Infected larvae of P. cynthia × ricini, T. asigna, D. trima and N. cytherea capensis all contained very similar virus particles which could not be distinguished under the electron microscope (Fig. 1) and had a diam. of about 35 nm. The virus material from D. trima and N. cytherea capensis contained particles of two different sizes whereas the virus particles of P. cynthia × ricini and T. asigna sedimented as a single component both in a sucrose gradient and in a CsCl gradient, and appeared homogeneous by electron microscopy. The D. trima mixture resolved into two distinct zones when centrifuged in a sucrose gradient. The upper zone mostly contained particles with a diam. of 27 nm while beneath it lay a more clearly defined zone containing particles with a diam. of 35 nm. These particles differed widely in buoyant density when centrifuged to equilibrium in CsCl, the 27 nm particle concentrating in a narrow zone at a density of 1.35 g/ml and the 35 nm particle at a density of 1.29 g/ml. Similarly, partially pure extracts of NβV were also shown to contain mixed
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Fig. 2. (a) Immunodiffusion test in an agarose gel. Wells containing antigens surround the antiserum well. D = Darna virus, N = Nudaurelia virus, P = Philosamia virus, T = Thosea virus, TA = Thosea virus antiserum. (b) Diagrammatic representation of precipitin lines shown in (a).

Table 2. Comparative complement fixation titres of antisera

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>TV</th>
<th>PV</th>
<th>DV</th>
<th>NβV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV</td>
<td>1/5120</td>
<td>1/320</td>
<td>1/1280</td>
<td>1/80</td>
</tr>
<tr>
<td>PV</td>
<td>1/160</td>
<td>1/5120</td>
<td>1/640</td>
<td>1/1280</td>
</tr>
<tr>
<td>DV</td>
<td>1/640</td>
<td>1/160</td>
<td>1/5120</td>
<td>1/2560</td>
</tr>
<tr>
<td>NβV</td>
<td>1/160</td>
<td>1/320</td>
<td>1/2560</td>
<td>1/5120</td>
</tr>
</tbody>
</table>

* Concentrations were adjusted to 1.0 absorbance unit at 260 nm.

populations of virus particles by equilibrium centrifugation in CsCl, and when material from a sucrose gradient was examined in the electron microscope, it was found that less than 1% of the particles had a diam. of about 27 nm and therefore differed noticeably from NβV. This contaminant, which, to judge from its size, is probably N. capensis δ virus (NδV) (Juckes, 1970), could be eliminated from the suspension by equilibrium centrifugation.

The viruses with particles of 35 nm diam. isolated from D. trima, P. cynthia × ricini and T. asigna, and described in this paper, will be called DV, PV and TV respectively.

The physico-chemical properties of the particles of each of the three viruses are shown in Table 1. For comparison, published data on the properties of NβV are also included, together with additional or supplementary data on relative mobility, polypeptide mol. wt. and the ratio $E_{260/280}$.

Virion polypeptides were compared, and their sizes estimated, by PAGE in 8% acrylamide gels and were found to be closely similar but not identical. When NβV and TV polypeptides were co-electrophoresed (on the same gel) two closely spaced bands were resolved and the mol. wt. of these were estimated as 62 100 (s.e. mean ± 800) for NβV and 59 900 (s.e. mean ± 300) for TV. In Table 1, the mol. wt. given for NβV and TV polypeptides are the means of the two sets of determinations. With the other viruses, neither the polypeptides of NβV + PV nor of PV + DV could be distinguished when each mixture was co-electrophoresed.

Fig. 2 shows the results of a gel immunodiffusion test where the four viruses were tested against TV antiserum. The formation of a spur of precipitate between each antigen well shows that all four viruses are serologically related to each other and yet each is serologically distinct. Furthermore, the direction of spur formation indicates that TV is closer to DV than to NβV and also that it is closer to PV than to NβV.
Since complement fixation (CF) is a more sensitive and reliable indicator of serological relationships than gel immunodiffusion, quantitative CF titres were determined for each combination of virus and antiserum. The results of these tests are shown in Table 2. If the reciprocal CF titres are taken together, then it is possible to assess the degree of serological relationship between the viruses. The most closely related viruses are NflV and DV, followed in decreasing order by TV and DV, NflV and PV, DV and PV, TV and PV and NflV and TV. These results amplify and confirm those obtained in the gel immunodiffusion test.

DISCUSSION

Our experiments show that DV, NflV, PV and TV share most of their properties. The finding that the particles of PV contain RNA (Table 1) is at variance with an earlier report on the properties of this virus (Longworth & Harrap, 1968). These authors described a virus isolated from four saturniid species, including P. cynthia × ricini. This virus had particles with a diam. of about 37 nm, a sedimentation coefficient of 200S and it was reported that they contained DNA rather than RNA. The original material from which Longworth & Harrap (1968) prepared PV particles, as well as a specific antiserum, was still available, and so it was decided to re-examine some of the properties of this culture. The original antiserum reacted with our PV particles which we showed to contain RNA and not DNA (Table 1). When particles of Longworth and Harrap's isolate of PV were purified and their nucleic acid type determined colorimetrically, the diphenylamine test proved negative and the orcinol test positive. It is apparent, therefore, that this virus contains only RNA, and it would seem that the original determination was in error.

Since only single determinations were made of the sedimentation coefficients of DV, PV and TV, no significance can be attached to apparent differences in the values for s given in Table 1. However, all, including that for NflV, lie within a reasonably narrow range (194 to 210S).

The buoyant density in CsCl, percentage RNA, the ratio $E_{260}/E_{280}$ and relative electrophoretic mobility of the particles of the four viruses show some degree of correlation. Thus, NflV and DV which have the densest particles also contain slightly more RNA than the lightest two, which is reflected in a higher ratio $E_{260}/E_{280}$. Their greater relative electrophoretic mobility points to a greater net-negative charge. This difference in charge, together with differences in RNA content, may contribute to their greater buoyant density by the binding of caesium ions.

Although no direct determinations were made of the mol. wt. of DV, PV and TV, it is possible to obtain an estimate from the mol. wt. of the capsid polypeptide and the proportion of protein in the particle. If we assume that the particles contain 240 copies of the polypeptide and consist of RNA and protein only, then the mol. wt. of the particles is equal to 240 x mol. wt. polypeptide/fraction of protein in the particle. Using the experimentally determined values given in Table 1, a mol. wt. of 16·9 x 10$^6$ is obtained for NflV, 16·8 x 10$^6$ for DV, 16·6 x 10$^6$ for PV and 16·2 x 10$^6$ for TV. Similarly, by subtracting the mol. wt. of protein from that of the whole particle a value for the mol. wt. of the RNA can be obtained. This yields a value of 1·9 x 10$^6$ for NflV, 1·9 x 10$^6$ for DV, 1·6 x 10$^6$ for PV and 1·7 x 10$^6$ for TV. The values for NflV and its RNA are no more than 4% higher than those given by Polson et al. (1970) and Struthers & Hendry (1974), and are therefore broadly comparable.

The results presented in this paper, together with the published data for NflV, are sufficient to enable a comparison to be made between this group of four viruses collectively and other virus groups. It is clear that, like the caliciviruses, these insect viruses are distinct from the
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mammalian picornaviruses as constituted by the two recognized genera Enterovirus and Rhinovirus (Melnick et al. 1974). The caliciviruses have provisionally been set aside from the picornaviruses principally on account of their polypeptide composition. Thus, both enteroviruses and rhinoviruses contain four major polypeptides in their capsids, derived by post-translational cleavage from a large precursor protein. The caliciviruses, by contrast, contain a single polypeptide species in their capsid and recent evidence suggests that it results from translation of a monocistronic mRNA (Black & Brown, 1976).

In considering size and polypeptide composition alone there is an obvious similarity between the caliciviruses and the four insect viruses described in this paper. However, when the two groups are compared on the basis of other key properties (Table 3) major differences are immediately obvious. Thus, particles of the caliciviruses, although they possess about twice as much RNA as those of the insect viruses, have a considerably lower mol. wt. This is because their capsids contain only three-quarters of the weight of protein possessed, for example, by NfV. This difference, which is in the order of about $3.6 \times 10^6$, and which contributes significantly to the lower range of S values for the caliciviruses is a result of a difference in their structure; both are icosahedra, but the triangulation number (T) of the caliciviruses is 3, and that of NfV and related viruses is 4.

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