Kelp Fly Virus

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SUMMARY

A virus from adult kelp flies (Chaetocoelopa sydneyensis) has been cultured in
larvae of the waxmoth (Galleria mellonella). This virus has isometric particles that
are 29±1 nm in diameter and resemble reovirus particles in appearance. Some
chemical and physical properties of these particles have been determined. They
contain single-stranded RNA, but have a unique set of the properties of the par-
ticles reported for other picornaviruses, and differ from all in surviving for 10 min
at 90 °C but not 100 °C. The cryptogram of kelp fly virus is R/1:3:5*:S/S:1/*.

INTRODUCTION

Adult kelp flies (Chaetocoelopa sydneyensis Schin.; Diptera, Phycodromidae) were
collected from rocks at Wapengo on the South Coast of New South Wales, Australia. The
larvae of these flies feed in kelp lying on beaches, and the adults congregate during winter
in large numbers on the spindrift-moistened undersurface of overhanging rocks near the
high water level. A water extract of about 1 × 10³ apparently healthy flies collected from one
such cluster containing about 2 × 10⁵ individuals was examined in the electron microscope,
but no virus-like particles were found. The extract was then injected into grubs of the
greater waxmoth (Galleria mellonella), and after 4 days all the grubs had died. Extracts of
the dead grubs contained isometric virus-like particles, which were not found in extracts
of comparable uninoculated grubs. We have shown that these are the particles of a virus
which we call kelp fly virus (KFV) and which has subsequently been isolated from kelp
flies collected from several coast sites within 250 km of Canberra. In this paper we report
some properties of KFV, which seems to be distinct from any other previously described
virus.

METHODS

Virus. As stated above, kelp flies were tested for KFV by injecting water extracts of them
into the haemocoel of Galleria mellonella grubs and subsequently the virus was bioassayed
or grown for study, by passaging in G. mellonella. Extracts of insects either for inoculum,
or for virus purification, were prepared by homogenizing the insects in 0.05 M-ammonium
acetate and carbon tetrachloride (about 0.1 g insect/ml/ml). The emulsion was centrifuged
at 8000 g for 5 min and the aqueous phase collected. For this work penultimate instar grubs
were used, and were infected by injecting each with about 2–5 μl of inoculum through a
proleg. For virus purification the inoculated grubs were kept for 3 days at 30 °C and
either used immediately or stored at −20 °C. In bioassay tests the grubs were kept for
4 days at 30 °C and the dead removed and recorded daily.
Virus purification. Extracts of infected grubs were prepared, as described above, except that a few crystals of phenylthiocarbamide were added to suppress melanin formation. Each extract was then rehomogenized several times with carbon tetrachloride to remove lipid, and the virus particles sedimented either by centrifuging at 120,000 g for 1 h or by adding polyethylene glycol (mol. wt. 6000) to 10% (w/v) and sodium chloride to 0.5 M and centrifuging at 5000 g for 20 min. Sedimented virus particles were resuspended in 0.05 M-ammonium acetate, clarified by centrifuging at 8000 g for 10 min, and the suspension layered on a 10 to 40% (w/v) sucrose gradient and centrifuged for 2 h at 90,000 g. The light-scattering band was collected, and after dilution or dialysis the virus particles were sedimented and concentrated as described above. The particles were further purified by centrifuging in a caesium chloride gradient in neutral 0.01 M-tris for 16 h at 90,000 g. The light-scattering band was collected, dialyzed against 0.05 M-ammonium acetate, concentrated as above, and stored at 4 °C.

Serology. Antisera were prepared by injecting rabbits intramuscularly twice at a fortnight's interval with virus preparations (about 2 mg virus/injection) emulsified in Freund's complete adjuvant.

Serological tests were done either by the two dimensional diffusion-in-gel method (Mansi, 1958) or by titration in mixed liquids (Bawden, 1956).

Stability tests. The stability of the virus at different temperatures was measured using purified virus preparations diluted to an $E_{260}$ of 1.0 and then further diluted 100-fold with distilled water or neutral solutions of various salts. Portions were heated at different temperatures for 10 min in screw-cap glass tubes, cooled rapidly in tap water and then stored in ice until tested for infectivity.

The acid stability of the virus particles was also measured using preparations diluted similarly into 0.1 M-acetate buffer at various pH values. After 15 min at room temperature the samples were mixed with 9 vol. of neutral 0.2 M-tris buffer, and their infectivity tested.

The rate of inactivation of KFV particles by u.v. light was estimated using a purified preparation at 1 $E_{260}$/ml (1 cm path) which was then diluted $1 \times 10^{-6}$-fold with 0.05 M-ammonium acetate. 1.5 ml samples were irradiated for different periods in glass Petri dishes 30 cm from a portable u.v. lamp (Mineralight 144W). The samples were diluted with 3 vol. of buffer and their infectivity tested.

Biochemical analyses. KFV preparations were tested for nucleic acid by the orcinol and diphenylamine methods (Schneider, 1957). Similar preparations were used to determine the base composition of KFV nucleic acid by the acid hydrolysis and chromatography method of Markham (1955), except that the chromatographic separation was done on 0.25 mm thick layers of Machery and Nagel MN 300 cellulose using an isopropanol:water:HCl (70:24:6) solvent.

For the estimation of amino acid composition of virus particles, preparations were hydrolysed for 24 h in performic acid, and the resulting amino acids separated and assayed in a Beckman amino acid analyser.

Biophysical analyses. The sedimentation coefficient ($s_{20, w}$) of KFV particles was estimated using a Beckman Model E ultracentrifuge or an M.S.E. Centriscan. Their density was determined in CsCl solutions also by centrifugation. Approx. 0.05 O.D. units of purified virus particles were mixed with caesium chloride in 0.01 M-tris and were centrifuged at 44,000 rev/min at 20 °C. The initial density of the CsCl solution was measured in a Zeiss refractometer at 25 °C and corrected to 20 °C using the relationship $\rho_{20} = 138.04/(138.11 (1/\rho_{20}) - 0.38)$ derived from published data (Dawson et al. 1969). After centrifuging for 16 h the cells were scanned at 260 or 254 nm, and the densities of the peaks calculated using the
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point on the gradient at which the initial density occurs, as a reference marker (the ‘hinge point method’), as described by Ifft, Voet & Vinograd (1961).

The KFV genome was extracted from particles suspended in 0.1 SSC by shaking twice with phenol saturated with 0.1 SSC and then several times with ether. The strandedness of the genome in 0.1 SSC was assessed by measuring its thermal hyperchromicity when heated from 20 °C to 85 °C, at a rate of 1 °C/min, using Escherichia coli DNA for comparison. The size of the genome was estimated for us by Russell Regnery by comparing its rate of electrophoretic migration in 1.5% agarose gels (Davey, 1973) with that of the Semliki Forest virus and tobacco mosaic virus RNA genomes, which are 4.3 and 1.6 × 10⁶ and 2.0 × 10⁶ daltons respectively.

The size and number of proteins in KFV particles was also assessed by comparing their electrophoretic mobilities with those of various standard marker proteins in 5 to 15% polyacrylamide gels (Weber & Osborn, 1969).

Electron microscopy. Virus samples were mixed with 4% aqueous sodium silicotungstate, dried on carbon-coated grids and examined in a Philips 301 electron microscope. The magnification of the micrographs (about × 60,500) was calibrated using the 8.6 nm spacing of fixed catalase crystals in separate micrographs.

RESULTS

Effect of temperature on yield of KFV

The virus content of extracts of inoculated grubs kept at different temperatures was estimated by serological titration which showed that after 3 days more virus was obtained at 30 °C than at higher or lower temperatures (15.5, 20, 26, 34.5 °C) though the yield at 26 °C was similar to that at 30 °C; at 30 °C, 1 g of grubs yielded about 70 μg of virus.

Infectivity of KFV

The infectivity of a range of dilutions of KFV preparations were estimated as described above.

KFV particles probably have a slightly greater mass than poliovirus particles (9 × 10⁶ daltons; Cooper et al. 1971) because they have a larger RNA (see below); we assume a particle mass of about 1 × 10⁷ daltons. One can assume by analogy with other viruses (Gibbs & Harrison, 1975) that the extinction of KFV particles in 260 nm wavelength light (1 cm path length) should be about 7 O.D. units/mg/ml. The infectivity experiments indicated an LD₅₀ of approx. 2000 particles/grub.

The physical properties of KFV particles

Purified preparations of KFV contained many isometric particles about 29 ± 1 nm in diam., which were not found in preparations of uninfected grubs.

High resolution electron micrographs of these particles showed that they are strikingly similar to the ‘cores’ of reovirus particles (Luftig et al. 1972), though the latter were much larger (52 nm diam.). Like the reovirus cores, the KFV particles had surface projections apparently located on fivefold icosahedral axes (Fig. 1). These projections, sometimes seen end-on (Fig. 1; b2–b5), protruded some 8 nm from the virion surface with a hollow bell-like shape about 8 nm diam. at the inner and increasing to about 12.5 nm at the outside end. Separate brick-shaped subunits (Fig. 1; d₄ and d₅) were usually also found in the preparation and these also measured about 8 × 12 nm. However it is not clear what relation these
have to the KFV particle, nor whether they originate from a missing outer layer of capsomers like that which is found in reovirus particles.

In the analytical centrifuge, KFV particles sedimented as a single component; its mean sedimentation coefficient (two estimates on each of three preparations) was 158S.

The apparent density ($\rho_{20}$) of KFV particles in CsCl was $1.425 \pm 0.002$ at pH 7, $1.430$ at pH 8 (only one determination), and $1.467 \pm 0.005$ at pH 9. The apparent density thus increased with pH over the range tested. Similar increases of density in CsCl have been observed for bee acute paralysis virus (Newman et al. 1973) and for FMDV (Rowlands, Sangar & Brown, 1971). This increase in density may result from a slow interaction between the virus particles and the CsCl; in one experiment at pH 7, the density increased from $\rho = 1.418$ 12 h after the start of centrifuging to a stable value of $1.423$ after 17 h. This result is similar to that for FMDV as reported by Rowlands et al. (1971). Purified preparations of KFV particles had u.v. absorption spectra typical of nucleoproteins. They had a clear peak in extinction at 259 nm wavelength and the mean $E_{260}/280$ ratio of 20 preparations was 1.65 (uncorrected for light-scattering).

The composition of KFV particles

Chemical tests showed that KFV particles contain RNA, and no detectable DNA, but these tests do not indicate whether KFV RNA is single- or double-stranded. To distinguish between these possibilities, RNA from the particles was thermally denatured and its hyperchromicity measured (Fig. 2). There was a gradual increase in extinction of about 20 %
Fig. 2. Effect of temperature on the optical density of KFV RNA and *E. coli* DNA. 
\[\text{---}, \text{KFV RNA}; \text{--}, \text{E. coli DNA.}\]

Fig. 3. Relative positions of RNAs in agarose gels plotted against the mol. wt. of RNA.
between 20 °C and 70 °C, which indicates that the RNA is principally single-stranded, with about 40% of its bases paired.

The size of the RNA extracted from KFV particles was estimated electrophoretically in 1.5% agarose gels to be about 3.5 × 10⁶ daltons (Fig. 3).

The product obtained by acid hydrolysis of KFV particles or of extracted RNA gave four u.v. absorbing spots when fractionated on thin cellulose layers. These spots had Rf values close to those reported by Markham (1955) for guanine, adenine, cytidylic acid and uridylic acid, and when eluted were found to have the absorption spectra of these compounds. The mean base ratio of four such determinations was: guanine 18.8 ± 1.6; adenine 34.0 ± 1.3; cytosine 18.6 ± 0.9; uracil 28.6 ± 0.5. The base ratios also provide further evidence that the RNA is single-stranded.

KFV particles were chemically disrupted and the resulting separated proteins analysed by electrophoresis in polyacrylamide gels. Two major proteins were found and these had average estimated mol. wt. of 73,000 ± 700 and 29,400 ± 750 (mean of six analyses) (Fig. 4). The optical densities of these two proteins in stained gels were in the ratio 1 : 2 respectively. Hence, assuming that these proteins fix stain in amounts determined solely by their mass, they are present in KFV particles in a 1:5 molar ratio.

Several minor protein species were also obtained even when the proteins had been carboxymethylated before analysis and these were particularly well separated in the 12% polyacrylamide gels (Fig. 5). We do not know whether these minor species are artefacts, minor components of the particles or absorbed contaminant proteins.

The amino acid composition (moles %) of the unfractionated proteins of KFV particles was: ala, 8.1; arg, 3.2; asp, 18.6; cysteic acid, 1.7; glu, 8.9; gly, 8.0; his, 0.4; ile, 5.7; leu, 9.2; lys, 4.7; met sulphone, 2.2; phe, 0.15; pro, 7.5; ser, 6.2; thr, 6.1; trp, not determined; tyr, 0.4; val, 8.9. These data were analysed by the FITMOL method (Gibbs & McIntyre,
There was no clear evidence from this analysis that the protein contained an integral number of amino acids between 100 and 1000 residues. This suggests either that the protein of the particle contains more than 1000 amino acids, or that it contains no amino acids present as 1 to 3 molecules per polypeptide, or, more likely, that it is a protein mixture whose constituents are not present in integral molar amounts.

**Stability of KFV particles**

KFV particles are unusually resistant to heat, and retained infectivity even after 10 min at 90 °C though not after 10 min at 98 °C or after autoclaving for 10 min. Furthermore there was no detectable loss of infectivity at 80 °C, though in some experiments there was usually some loss at 90 °C, indicating a large temperature coefficient (Q10) of inactivation.

Magnesium sulphate or sodium chloride had no detectable effect on the heat stability of KFV (Table 1); however infectivity tests on KFV heated in magnesium chloride solutions at 80 °C showed that the salt decreased virus stability at least 10⁶-fold.

There was no detectable effect of pH on the infectivity and stability of KFV particles between pH 3 and pH 7.

Ultraviolet irradiation of KFV preparations also indicated, like the dilution experiments, a first-order inactivation and hence that the genome of KFV is probably not divided between several particles.

**DISCUSSION**

The virus we have isolated appears to cause no symptoms in kelp flies. It could not be detected by electron microscopy of an extract of the flies. The particles replicate in *Galleria mellonella* larvae and were initially detected only in wax moth larvae injected with an extract.
Table 1. Some physico-chemical properties of picornavirus particles*

<table>
<thead>
<tr>
<th>Virus</th>
<th>S value</th>
<th>Accessory particles</th>
<th>Density × 10^{-6}</th>
<th>Stranded-ness</th>
<th>RNA</th>
<th>Proteins</th>
<th>Thermal inactivation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Number</td>
<td>Sizes × 10^{-3}</td>
</tr>
<tr>
<td>KFV</td>
<td>158</td>
<td>-</td>
<td>1'42</td>
<td>c. 3.5</td>
<td>1</td>
<td>19</td>
<td>34 34 29</td>
</tr>
<tr>
<td>Bee acute paralysis</td>
<td>160</td>
<td>+</td>
<td>1'34</td>
<td>c. 2</td>
<td>1</td>
<td>19</td>
<td>30 21 30</td>
</tr>
<tr>
<td>Cricket paralysis</td>
<td>167</td>
<td>+</td>
<td>&gt;2</td>
<td></td>
<td>1</td>
<td>21</td>
<td>32 18 30</td>
</tr>
<tr>
<td>Sacbrood</td>
<td>157</td>
<td>-</td>
<td>1'33</td>
<td>2.6</td>
<td>1</td>
<td>19</td>
<td>32 18 31</td>
</tr>
<tr>
<td>Arkansas bee virus (major component)</td>
<td>138</td>
<td>-</td>
<td>1'37</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D. 5</td>
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<tr>
<td>Bee virus X</td>
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<td>-</td>
<td>1'36</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D. 5</td>
</tr>
<tr>
<td>Bee slow paralysis</td>
<td>175</td>
<td>+</td>
<td>1'35</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D. 5</td>
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<tr>
<td>Poliovirus</td>
<td>160</td>
<td>+</td>
<td>1'34</td>
<td>2.6</td>
<td>1</td>
<td>25</td>
<td>29 22 25</td>
</tr>
<tr>
<td>EMC</td>
<td>160</td>
<td>+</td>
<td>1'33</td>
<td>2.6</td>
<td>1</td>
<td>24</td>
<td>27 24 25</td>
</tr>
<tr>
<td>FMDV</td>
<td>146</td>
<td>-</td>
<td>1'43</td>
<td>2.6</td>
<td>1</td>
<td>24</td>
<td>26 28 22</td>
</tr>
<tr>
<td>Human rhinovirus</td>
<td>150</td>
<td>-</td>
<td>1'40</td>
<td>2.6</td>
<td>1</td>
<td>20</td>
<td>34 20 26</td>
</tr>
</tbody>
</table>

* The information in this Table is from this paper and also from Bailey & Woods (1974); Bellett (1967); Brown & Hull (1973); Brown, Newman & Stott (1970); Burness, Fox & Pardoe (1974); Butterworth (1973); Newman et al. (1973); Reinganum (1973); Rowlands, Sangar & Brown (1971); Vande Woude, Swaney & Bachrach (1972); Vanden Berge & Boeyé (1972); Wallis, Melnick & Rapp (1965); Wildy (1971).  
† Not determined.
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from kelp flies. No similar particles were detected in uninfected grubs or from grubs injected with extracts of other insects. KFV has also been isolated on other occasions from kelp flies obtained at different localities several months after the original collection. These facts strongly suggest that the virus described in this paper does come from the kelp flies and is not a virus carried latent in G. mellonella grubs. As yet we have no information on the ecology of KFV, though it seems to be most common in adult kelp flies in winter when they are gregarious.

REFERENCES


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