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Queensland fruit fly virus, a probable member of the Picornaviridae

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Summary. A picornavirus was isolated from various life stages of the Queensland fruit fly, *Dacus tryoni*. This virus, Queensland fruit fly virus (QFFV) has virions with a diameter of 30 nm and a sedimentation coefficient of 178 S. One third of the particles in preparations were empty capsids or natural top component (NTC) with a sedimentation coefficient of 95 S. The buoyant density (ρ) of virions and NTC in CsCl was 1.34 and 1.30 g/ml respectively; small amounts of a dense component (ρ = 1.45 g/ml) were also detected. The capsid contained three major protein species of molecular weight (mol.wt.) 41,700, 36,500, and 31,300, in approximately equimolar proportions. NTC contained three major species of mol. wt. 44,700, 41,700, and 31,300. The nucleic acid present only in the bottom component virions was RNA and comprised about 30% of the particle weight and had a mol. wt of 2.88 kd, contained a poly(A) tract, and had a base ratio: G = 20; A = 32; C = 15; U = 33. The mol. wt. of the virion was estimated to be ≈9.5 kd. When virions were heated at 56 °C and above, they converted into artificial top component (ATC), which had the same protein composition as the virion when analysed by SDS-PAGE. In immunodiffusion tests the virions and NTC were indistinguishable, but a minor difference in antigenicity was detected between the virions and ATC. Virions were stable between pH 3 and 9 inclusive, and between 5 and 7 in the presence of 0.14 M NaCl. Immunodiffusion tests showed that QFFV was serologically unrelated to a range of picornaviruses as well as an unclassified virus isolated from the Mediterranean fruit fly, *Ceratitis capitata*. The data show that QFFV is probably a member of the *Picornaviridae*, genus Enterovirus.

Introduction

The Queensland fruit fly, *Dacus tryoni* (Froggatt), has been a pest of cultivated fruit in Queensland since 1850, and, during those 100 years has become established throughout southeastern Australia.

Since 1946, several measures have been taken to control the further spread

of the fly. These include the spraying of all infested trees with DDT, and the installation of border road blocks to stop the passage of fly-infested fruit. These measures have now been abandoned. Since 1980, the only attempts to control the fly populations have been to distribute protein-malathion baits in the major fruit growing areas.

In this paper we report experiments to characterize a virus obtained from a culture of *D. tryoni*. This culture was originally derived from a laboratory culture of the fly maintained at the School of Zoology, University of New South Wales. A cytoplasmic inclusion virus of these flies was reported by Moussa [16]. This virus induces premature mortality and decreased fecundity, symptoms which have also been noted in our fly culture at the Plant Research Institute, Burnley. Moussa's report detailed effects of the virus on the flies and its intracellular location, this paper describes the isolation and detailed properties of the virus, which we have named "Queensland fruit fly virus" (QFFV).

Methods

Fly stocks

D. tryoni were bred at the Fly Suppression Unit of the Plant Research Institute as part of a "sterile male release" program. Pupae and flies were the kind gift of Mr. G. Betlinski.

Extraction and purification of virus

Flies or pupae were homogenized in 6 volumes of chilled PMB (2 mM-sodium phosphate buffer, pH 7.5 + 1 mM-$MgSO_4 \cdot 7H_2O$ + 2 mM-phenylmethylsulfonyl fluoride (PMSF) + 10 mM-sodium diethyldithiocarbamate). The slurry was sonicated for 15 seconds at 50 W and filtered through two layers of cheesecloth. To the filtrate was added 0.25 volume of Mg-saturated bentonite [6] and mixed thoroughly. The suspension was then emulsified with 0.5 volume of CCl_4 and centrifuged at 27,000 **g** for 10 minutes. The aqueous layer was withdrawn, made 1% with respect to Triton X-100 and centrifuged at 200,000 **g** for 1 hour. The pellets were suspended in a small volume of 0.05 M-phosphate buffer, pH 7.5 (PB) and insoluble material removed by centrifugation at 27,000 **g** for 10 minutes. Virus was further purified by centrifuging into a 10–40% (w/v) sucrose gradient in PB at 104,000 **g** for 2.25 hours. The light-scattering zone was removed and the virions in it collected by centrifugation at 200,000 **g** for 1 hour and recycled in a second sucrose gradient.

Physicochemical properties

For electron microscopy, virion preparations were mixed with an equal volume of 0.5% uranyl acetate and examined using a JEOL JEM-100 S electron microscope. The size of QFFV particles was estimated by reference to those of tobacco mosaic virus, as previously described [22].

The sedimentation coefficient ($S_{20,w}$) was determined by rate-zonal centrifugation of purified QFFV virions into a linear 10–40% (w/w) sucrose gradient in PB at 200,000 **g** for 2.5 hours. ^{3}H-labelled virions of cricket paralysis virus (CrPV), which have a S value of 167 [20] was co-sedimented as an internal reference. Fractions were collected by bottom puncture of the tube and the radioactivity and protein concentration of each fraction determined by scintillation counting and the Bradford method [1] respectively. As a check on the linearity of the gradient, the sucrose concentration of each fraction was measured, using an Abbe refractometer.

The buoyant density of QFFV virions were determined in a 1.1–1.5 g/ml gradient of CsCl in PB. A suspension (0.2 ml) of purified virions was layered onto the preformed gradient and centrifuged at 200,000 **g** for 16 hours at 10 °C. Fractions were collected by bottom puncture and the protein concentration of each fraction determined by the Bradford method [1]. The refractive index of each fraction was measured and the values converted into density using the formula of Vinograd and Hearst, as given in Brakke [2].

Ultraviolet absorption spectra of virion preparations were measured in a Pye-Unicam spectrophotometer. The solvents were 0.125 M-Tris-HCl, pH 6.8 (virions and capsids) and 10 mM-Tris-HCl, pH 8.0 + 1 mM-EDTA + 0.1 M-NaCl (RNA).

For the determination of the chemical composition of QFFV virions, a standard amount ($OD_{260} = 3.16$) was analysed quantitatively for protein by the method of Lowry et al. [13] and for RNA by the method of Mejbaum [15].

Virion proteins were dissociated and analysed by SDS-PAGE [10], using an 11 or 15% (w/w) vertical slab gel beneath a 4% (w/w) stacking gel. A mixture of standard proteins, consisting of phosphorylase B, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were electrophoresed in parallel as molecular weight (mol. wt.) markers. Gels were fixed in acetic acid : methanol : water (10 : 40 : 50 v/v/v), stained in the same solvent containing 0.1% Coomassie Blue R 250 and 0.1% cupric sulphate and de-stained in 5% acetic acid + 4% methanol.

RNA was extracted from QFFV virions by digestion with proteinase K, as described by Crump and Moore [5]. Ethanol-precipitated RNA was denatured in formamide and electrophoresed in a 0.8% agarose gel containing formaldehyde [11]. Gels were fixed in 10% acetic acid, stained in 0.2% methylene blue in acetate buffer (0.4 M-sodium acetate, pH 7.4) and destained in several changes of tap water. TMV genomic RNA (mol. wt. 2.1 kd), HeLa cell 28 S and 18 S ribosomal RNA (mol. wt. 1.75 and 0.67 kd respectively) were extracted, denatured similarly and used as mol. wt. standards.

The base ratio of QFFV genomic RNA was determined by the chromatographic method of Markham [14].

For the detection of poly(A), a prepacked oligo(dT)-cellulose type 7 column was used (P-L Biochemicals, Redi-cols, Pharmacia). The column was washed with 10 mM-Tris-HCl (pH 7.5), followed by 5 ml of 50 mM-KCl + 10 mM-Tris-HCl (solution A). Viral RNA was then layered onto, and allowed to enter the column, followed by 5 ml of solution A. Attached poly(A)-RNA was eluted with 5 ml of 0.1 M-KCl + 10 mM-Tris-HCl (solution B). Fractions (0.6 ml) were collected from the addition of the sample till the end of elution with solution B and assayed for protein and RNA by the methods of Bradford [1] and Mejbaum [15] respectively. The column was washed with 3 volumes of solution A after use.

Stability of the virions under various conditions

Thermal stability was tested by heating aliquots of purified virions in 50 mM-phosphate buffer (pH 7.2) at 50, 55, 60, and 65 °C for 15 min. The suspensions were examined in the electron microscope and also tested for antigenic stability by immunodiffusion in agarose.

The effect of pH on the virions was tested by mixing 0.1 ml of a virion preparation in distilled water with an equal volume of 0.1 M-phosphate-citrate buffer of a particular pH, which ranged between 3 and 9 inclusive, at intervals of one unit. The suspensions were incubated for 1 hours at 37 °C and examined in the electron microscope. A similar experiment, designed to test the stability of the virions in halide, consisted of mixing 0.1 ml of a virion preparation in distilled water with an equal vol of 0.1 M-phosphate-citrate buffer (pH 5.2, 5.6, 6.4, and 7.0) containing 0.28 M-NaCl. After incubation for 30 minutes at 37 °C, the mixtures were diluted with an equal volume of distilled water and examined in the electron microscope.

Serology

An antiserum to QFFV virions was prepared by immunisation of a rabbit. Purified virus and Freund's complete adjuvant (50 : 50) was injected into each hind leg. The injections were repeated after four and six weeks. Two weeks later, blood was withdrawn by cardiac puncture. The antiserum titre was determined by microprecipitation.

QFFV virions were tested by immunodiffusion (ID) against antisera to a range of insect picorna- or picorna-like viruses and by immune electron microscopy (IEM) against a range of mammalian picornaviruses, by methods previously described [21].

To assay the presence and amount of virus in live and dead flies, radial immunodiffusion was used. Flies were ground in a volume of PMB proportional to the number of flies and the extracts were processed as already described, to the stage of high-speed centrifugation. The pellets were suspended in a small volume of buffer proportional to the original number of flies. A standard suspension of purified virions (1.325 mg/ml, as estimated from the extinction coefficient) was used to prepare a two-fold dilution series. Suspensions were allowed to diffuse into a 1.5 mm thick layer of 0.8% agarose containing 3% anti-QFFV serum and 0.5% sodium azide. After incubation at 37 °C for 24 hours, the diameters of the precipitin haloes were measured and virions concentrations estimated by reference to a curve prepared from the standards.

Cells

Drosophila melanogaster cell lines 1 and 2 [24] and *Spodoptera frugiperda* cell line (Sf9) [26], were a gift of Dr. Paul Scotti, and *Heteronychus arator* cells [4] of Dr. Alan Crawford, both of DSIR, Auckland, New Zealand. The cells were maintained in Schneider's *Drosophila* medium (GIBCO, N.Y.) supplemented with 10% inactivated foetal calf serum (CSL, Melbourne). *Aedes albopictus* C6–36 cells [8], BHK, Vero and HeLa cell lines were grown in Eagle's Minimal Medium, (MEM, Flow Laboratories Australasia Pty. Ltd.) supplemented with 10% foetal calf serum.

Results

Virus purification

Previous studies [22] have shown that the use of a bentonite-CCl_4-Triton X-100 clarification step in the purification of a number of insect picornaviruses is particularly useful in removing host material from virion preparations. In the present study, much host material was removed with this method. As a result, the virions banded as a discrete zone in sucrose density gradients.

Partially purified virions separated into two light-scattering zones after centrifuging into a sucrose density gradient. Electron microscopy showed that the faster-sedimenting zone, located near the centre of the gradient, contained intact virions and the slower-sedimenting zone contained "empty" particles. Following the suggested terminology of Rueckert [23], the latter will be referred to as "natural top component" (NTC). The proportion of NTC to intact virions, assessed by electron microscope examination preparations before sucrose gradient centrifugation, was of the order of 1 : 2. In an attempt to increase the yield of virus, by releasing intracellular virions, the crude extract was sonicated. Preparations made in this manner, when assessed by either optical density readings or by electron microscopy, showed an increase in the number of virus particles. The proportion of NTC to virions was not affected by this treatment.

When the first preparations of QFFV virions were analysed by SDS-PAGE, four proteins were consistently detected (Fig. 1). One, provisionally named VP3a, occurred in minor amounts and was probably a proteolytic cleavage product of a slightly larger parent protein (VP3). For this reason, the protease inhibitor PMSF was subsequently included in the extraction medium. It was shown that VP3a was derived by proteolysis from VP3 by incubating a purified virion preparation extracted in the presence of PMSF with an extract of dead flies. The incubated virions were centrifuged to equilibrium in a CsCl gradient and the capsid proteins analysed by SDS-PAGE. The results (Fig. 1) show clearly that, as a result of this treatment, VP3a increased at the expense of VP3.

All preparations of virus extracted from freshly dead flies, even in the presence of PMSF contained traces of VP3a implying that proteolysis occurs rapidly post mortem. As live flies also contained large quantities of virus, all further preparations were made either from live flies or from pupae just before emergence. Virus obtained from these sources contained no trace of VP3a and was used for the characterisation of QFFV virions and in serological tests.

Material from each stage of the fly's life cycle was examined for the presence of virions. Although none could be detected with certainty in freshly laid eggs, appreciable quantities were extracted from larvae, pupae, pupae which had aborted prior to ecdysis and from both live and dead flies. A radial immunodiffusion test, designed to quantify amounts of virus in live and dead flies showed that these contained 5.3 µg and 3.3 µg virus per fly respectively.

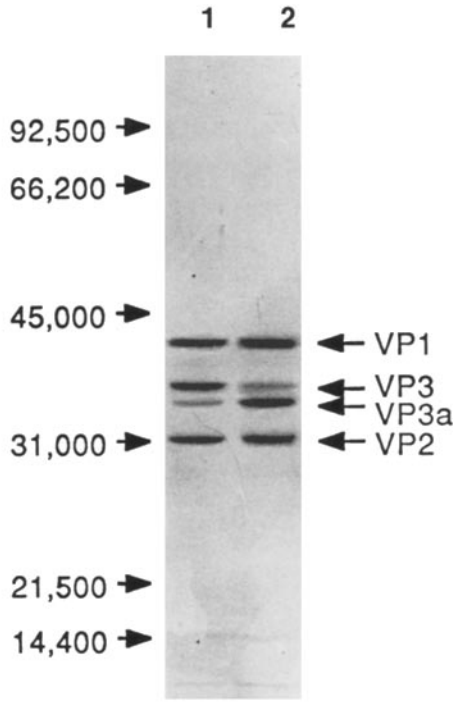

Fig. 1. SDS-PAGE analysis of the proteins of QFFV particles. Pure QFFV virions were incubated at 37 °C for 1 hour with a virus-free extract of flies. Proteins of both treated (*2*) and untreated (*1*) particles were analysed in a 11% gel and stained with Coomassie blue

Physicochemical properties

QFFV particles, negatively stained with uranyl acetate were isometric and uniform in size (Fig. 2). An average particle diameter of 30 nm was calculated by comparison with the diameter of 16 nm for TMV particles.

Rate-zonal centrifugation of virions and NTC into linear sucrose gradients was used to estimate sedimentation coefficients. Using CrPV virions ($S_{20,w} = 167$) as an internal reference, values of 178 and 95 S were obtained for virions and

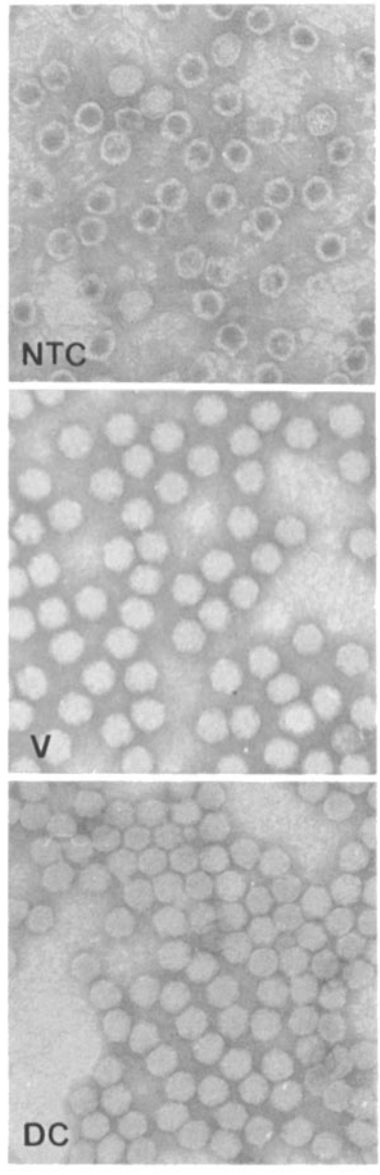

Fig. 2. Electron micrographs of samples corresponding to the protein peaks recovered after isopycnic centrifugation of QFFV virions. Particles were negatively stained with uranyl acetate. *NTC* Natural top component; *V* virions; *DC* dense component

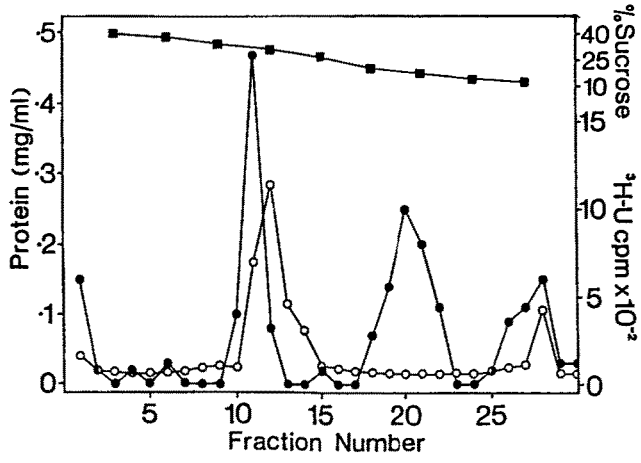

Fig. 3. Rate-zonal centrifugation of the virions of QFFV and ^{3}H-uridine-labelled CrPV into a sucrose gradient. Sedimentation was from right to left. ○ ^{3}H-uridine cpm; ● protein (mg/ml); ■ % sucrose

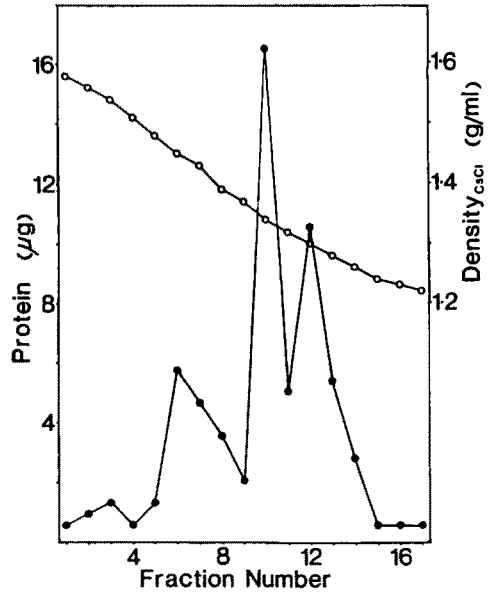

Fig. 4. CsCl density centrifugation of QFFV virions. Three peaks of protein were at densities 1.30 (NTC), 1.34 (V), and 1.45 (DC). Sedimentation was from right to left. ○ density CsCl (mg/ml); ● protein (µg)

NTC respectively (Fig. 3). From these values, an RNA content of 30% can be calculated [19].

When a mixture of virions and NTC were centrifuged to equilibrium in CsCl, they formed three bands with densities of 1.30, 1.34, and 1.45 g/ml (Fig. 4). Material from each band was examined in the electron microscope. It was found that the lightest particles were those of NTC and the other two consisted of intact virions (Fig. 2). The virions of density 1.45 g/ml are evidently "dense component" (DC), which has been detected in minor amounts in other picor-

naviruses [9, 28]. Virions, NTC and DC were antigenically indistinguishable by immunodiffusion.

The ultraviolet absorption data were used to calculate the $OD_{260/280}$ ratios of virion, NTC and RNA to be 1.655, 0.760, and 2.075 respectively. They are typical of a nucleoprotein (virion), protein (NTC), and nucleic acid.

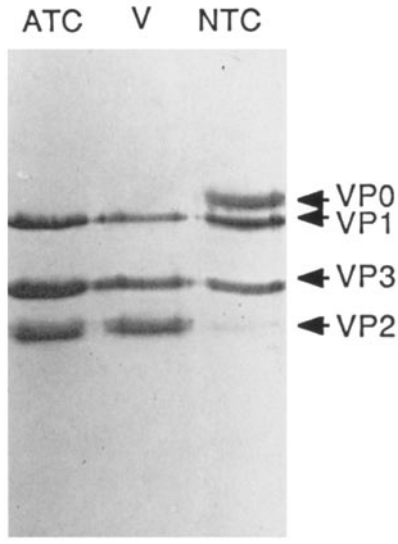

Fig. 5. SDS-PAGE analysis of particles of QFFV. NTC and V were recovered from sucrose gradients and ATC was generated by heating V at 56 °C for 30 minutes. A 15% gel was used and stained with Coomassie blue after electrophoresis

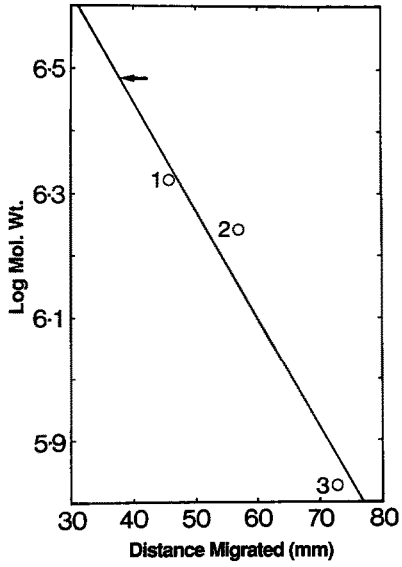

Fig. 6. Molecular weight estimation of QFFV virion RNA. The position of QFFV RNA is marked with an arrow (⬅). TMV RNA, 2.1×10^6 (*1*), HeLa 28 S RNA, 1.75×10^6 (*2*), and HeLa 18 S RNA, 0.67×10^6 (*3*) were used as markers. All RNA was denatured by formamide before agarose gel electrophoresis

QFFV virions contained 30.8% RNA, and from this value, an extinction coefficient ($E^{1\%}_{1\,cm}$) of 68 was calculated for the virus. This value was used to quantify the amount of virions in live and dead flies.

When analysed in a 15% gel, the capsid proteins of the virions separated as three species of mol. wt. 41,700, 36,500, and 31,300 (Fig. 5). NTC contained three major species of mol. wt. 44,700, 41,700, and 36,500, with a trace of the 31,300 mol. wt. species. Spectrophotometric quantification of dye eluted in 67% formic acid from stained gels indicated that VP1, VP2, and VP3 were present in approximately equimolar proportions.

The purity of the RNA extracted from QFFV virions is shown by its absorption data, as the $OD_{260/280}$ ratio exceeds 2.0. When electrophoresed into agarose gels under denaturing conditions, a mol. wt. of 2.88 kd was estimated for QFFV RNA (Fig. 6). The RNA was retained by an oligo(dT) cellulose column, indicating the presence of a poly(A) tract. The base ratio of the RNA was calculated from chromatographic analysis to be; G:A:C:U = 20:32:15:33.

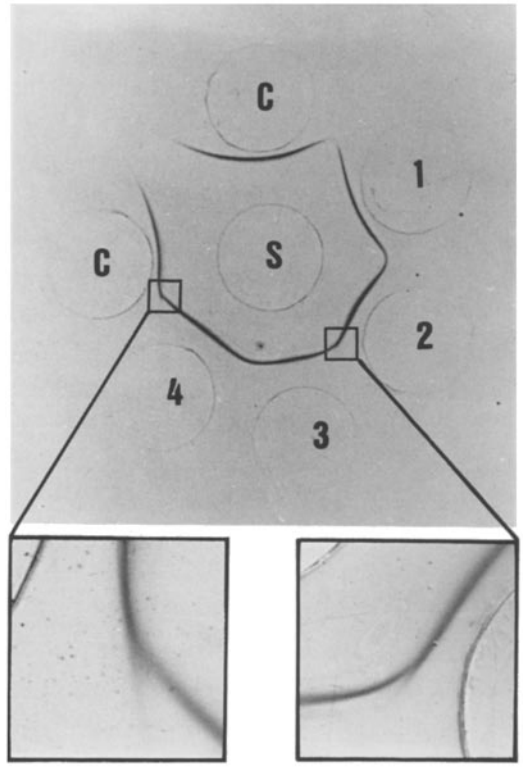

Fig. 7. Immunodiffusion of products obtained after heat treatment of QFFV. Antiserum to QFFV virions was in the centre well (*S*). *C* Wells contained untreated virions, *1–4* wells contained virions heated at 50°, 55°, 60°, and 65°C, respectively. Spur formation can be seen in the appropriate enlargements

Stability of the virus under various conditions

The thermal stability of QFFV was determined by heating aliquots of purified virions at various temperatures and then examining them in the electron microscope. The effect of heat was to convert virions into empty particles which, again, following the terminology of Rueckert will be referred to as "artificial top component" (ATC). The temperature at which 50% of the virions were converted to ATC was estimated as 57 °C. SDS-PAGE analysis of ATC showed that its protein were indistinguishable from that of the virions (Fig. 5). The transformation from virion to ATC was accompanied by a minor change in antigenicity since, when virions which had been heated at 55 °C and at 60 °C was reacted in neighbouring wells against an antiserum to the virion, a small precipitin spur developed (Fig. 7).

No morphological change was detected when virions were incubated at 37 °C for 1 hour over the pH range of 3 to 9 inclusive. Stability was also maintained at 37 °C for 30 minutes when virions were incubated in the presence of 0.14 M NaCl over the pH range 5.2 to 7.0.

Serology

QFFV virions reacted strongly with homologous antiserum and produced a sharp precipitin line when tested by immunodiffusion. The antiserum titre was 1/256.

The antigenic relationship of QFFV virions to those of other insect picorna- or picorna-like viruses was tested by immunodiffusion. Negative results were obtained with antisera to the following: bee slow paralysis virus, acute bee-paralysis virus, sacbrood virus, Kashmir bee virus, black queen cell virus, silkworm flacherie virus, termite paralysis virus, cricket paralysis virus, *Drosophila* C virus, *Drosophila* A virus, *Drosophila* P virus, and *Gonometa* virus [21]. When tested by immunoelectron microscopy, QFFV virions gave negative results with the following antisera pools: poliovirus 1, 2, and 3, Coxsackie A 1–24, Coxsackie B 1–6, Echovirus 2–9, 11–33, and 68–71. QFFV virions also failed to react with an antiserum to encephalomyocarditis virus (EMCV).

Of interest is a lack of antigenic relationship between QFFV and a virus of similar appearance isolated from the Mediterranean fruit fly, *Ceratitis capitata,* obtained from breeding stocks in Western Australia.

Growth of QFFV in vitro

One lepidopteran, one coleopteran, three dipteran and a range of mammalian cell lines were inoculated with QFFV. Culture fluids from each of these were passaged serially five times. On no occasion was any CPE observed, and no virions could be detected by immunoelectron microscopy.

Cross-transmission tests

Attempts to transmit QFFV to adult honey bees (*Apis mellifera*), and to the larvae of the wax moth (*Galleria melonella*) by injection, and, per os, to adults and larvae of *D. melanogaster* were unsuccessful.

Discussion

The results of these studies show that the properties of QFFV closely resemble those of the *Picornaviridae*. The morphology of its virions, capsid proteins sizes, the relation between NTC, ATC and virion, and the response of the virion to heat are all characteristic of this group of viruses. Where QFFV does differ is in its "overweight" nature when compared to other members of the group (Table 1). Hence the diameter of its particles, the sedimentation coefficient of both virion and NTC and the molecular weight of both the capsid and the RNA are all larger than those of poliovirus and EMCV.

The picornavirion typically contains four major species of capsid proteins VP1, VP2, VP3, and VP4, in order of decreasing mol. wt. The provirion, or NTC, contains three proteins—VP0, VP1, and VP3. On maturation, VP0 is cleaved to give VP2 and VP4. For this reason we conclude that the QFFV proteins of mol. wt. 36,500 and 31,300 represent VP3 and VP2, respectively, i.e., they migrate in reverse order. The same phenomenon has been noted in the case of hepatitis A virus [7, 27].

Although the data are incomplete, it is possible to obtain an estimate of the mol. wt. of the virion. Since VP1, VP2, and VP3 are present in near-equimolar amounts, then one can assume that the capsid contains 60 copies of each, in common with other picornaviruses. Therefore, the mol. wt. of the capsid is computed as 60 (41,700 + 36,500 + 31,300) = 6.57 kd. Since the mol. wt. of the RNA was experimentally determined as 2.88 kd, then the mol. wt. of the virion is 6.57 + 2.88 = 9.45 kd. The RNA content of the virus calculated from these values is 30.5%, which is very close to the value of 30.8% calculated from the

Table 1. Size-related properties of the virions of QFFV and two mammalian picornaviruses compared

	QFFV	Poliovirus[a]	EMCV[b]
$S_{20,w}$ virion	178	160	162.3
$S_{20,w}$ NTC	95	80	—[c]
Virion mol. wt. (kd)	9.45	8.0	8.5
Capsid mol. wt. (kd)	6.57	5.4	5.8
RNA mol. wt. (kd)	2.88	2.6	2.7

[a] Data from Rueckert [23]
[b] Data from Burness and Clothier [29]
[c] EMCV does not produce NTC

compositional analysis of the virus and to the value of 30% obtained from sedimentation coefficients using Reichman's formula [19].

With the exception of the cardioviruses, the virions of the mammalian picornaviruses can be converted into particles which are antigenically distinct from the virion. This conversion, from D to C antigenicity occurs, for example, when the virions are heated at about 56 °C and is accompanied by the loss of VP4 [23]. However, in the case of QFFV, the same treatment results in only a minor change in antigenicity (Fig. 7). Virions and NTC were antigenically indistinguishable by immunodiffusion tests so that, in this respect, they resemble the corresponding particles of echovirus 12 and foot-and-mouth disease virus but differ from those of poliovirus [23].

As regards the position of QFFV within the *Picornaviridae*, the pH stability data resemble those of the genus Enterovirus. Members of this genus typically produce NTC during the replication cycle and ATC when the virion is heated above a certain temperature. Also, their virions are stable under moderate salt conditions between pH 3 and 9 and are not affected by 0.14 M-halide over the pH range 5 to 7 [3]. QFFV conforms with all these criteria. However, although the genomic RNA contains a poly(A) tract, the low G+C content to that RNA is characteristic not of the mammalian but of those insect picornaviruses for which data are available [12].

While the studies reported in this paper were in progress a virus was also isolated from the Mediterranean fruit fly *Ceratitis capitata*. In the absence of appropriate antisera and antigen, there is no way of telling whether this isolate is related to that described by Plus et al. [18] and named the *Ceratitis* V virus. However, it was shown that the virions of QFFV are serologically distinct from those of a virus isolated from *C. capitata*.

Serological tests, using a range of antisera to both insect and mammalian picornaviruses, were designed to determine whether or not this is a new virus and not simply a variant of an existing one. The finding that the virions of CrPV and *Drosophila* C virus are serologically related [17, 21] and, likewise, CrPV and EMCV [25], points to the need to test virions of new isolates against all available antisera. However, we obtained no positive reactions in tests between QFFV virions and antisera to virions of 12 insect viruses and pooled antisera of mammalian picornaviruses. Within the limits tested, it does appear that QFFV is a new virus.

In summary, it is suggested that Queensland fruit fly virus is a new member of the *Picornaviridae*.

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