J. gen. Virol. (1980), **46**, 149–155
Printed in Great Britain

Small Virus-like Particles in Honey Bees associated with Chronic Paralysis Virus and with a Previously Undescribed Disease

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(*Accepted* 3 *August* 1979)

SUMMARY

Chronic bee-paralysis virus associate (CPVA) and cloudy wing particle (CWP) are about 17 nm in diam. contain RNA, have single polypeptide species and have indistinguishable buoyant densities in CsCl of about 1·38 g/ml; but they are serologically unrelated, have proteins of different mol. wt. and have significantly different sedimentation coefficients of 41S and 49S respectively. CPVA is significantly and possibly specifically associated with chronic paralysis virus but multiplies, relative to the paralysis virus, by different amounts in different tissues. CWP multiplies apparently independently.

INTRODUCTION

During investigations of chronic bee-paralysis virus (CPV), which has irregular anisometric particles, and of a previously undescribed disease of adult honey bees (*Apis mellifera*), we have isolated isometric particles of nucleoprotein about 17 nm in diam. The particles associated with the paralysis virus were first reported by Bailey (1976) and their replication may depend on some of the genetic information of the virus. Those associated with the previously undescribed disease, which we call 'cloudy wing', may be of an independent virus. The evidence for each possibility is circumstantial because experimental work has so far given equivocal results. Nevertheless, a description of the particles seems to be indicated since they are widespread and common in bees and could well cause confusion for other investigators.

METHODS

Virus purification

Chronic paralysis virus associate (CPVA). Adult bees were extracted in 4 vol. of 0·01 M-potassium phosphate buffer at pH 7·0 containing 0·02% sodium diethyldithiocarbamate (about 2 bees/ml) + 1 vol. diethyl ether. The mixture was emulsified with CCl_4, cleared at 8000 *g* for 10 min or at 3000 *g* for 30 min and then centrifuged at 75000 *g* for 3·5 h or at 100000 *g* for 2 h. The pellet was resuspended in 0·01 M buffer and after a further cycle of low and high speed centrifugation, was centrifuged down linear 10 to 40% (w/v) sucrose gradients in 0·01 M buffer for 4·5 h at 15 °C. Fractions were collected from the top of the tube, by displacement from beneath with 50% sucrose and assayed for A_{254} by means of an ISCO density gradient fractionator. Further purification for protein analysis was done in CsCl adjusted to the density of the particle and centrifuged in an MSE angle 10 × 10 ml rotor at 75000 *g* for 17 h at 15 °C. Fractions were collected by piercing the bottom of the tube and then assayed for A_{254}; u.v. absorbing fractions were chromatographed on a 20 ml

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column of Sephadex G25 (coarse). The first 4 or 5 ml after the void vol. contained most particles and were concentrated by freeze-drying.

Cloudy wing particle (*CWP*). Adult bees were extracted, either as described above for CPVA or with the concentration of phosphate buffer increased to 0·1 M both for the extraction and for the sucrose gradients.

Physico-chemical properties. Sedimentation coefficients ($s_{20,\,w}$) and buoyant densities in CsCl were determined in an MSE Centriscan centrifuge. Preparations were negatively stained with neutral sodium phosphotungstate and examined and photographed in a Siemens Elmiskop I electron microscope.

Tests for RNA and DNA were done by the orcinol (Ceriotti, 1955) and diphenylamine (Burton, 1956) quantitative reactions. As our estimates of virus concentration were based upon particle counts and u.v. absorption spectra and not upon dry weights, we have not calculated the percentage compositions. Analyses of virus proteins were done on 5% SDS-acrylamide gels using 16 well characterized protein markers in the mol. wt. range 10000 to 30000 (Carpenter *et al.* 1977). RNA was analysed on 2·8% acrylamide gels as described previously (Carpenter *et al.* 1977), except that agarose was not incorporated into the gel. Single stranded markers were used for mol. wt. calibration.

Relative amounts of CPVA, chronic bee-paralysis virus and CWP in sucrose and CsCl gradients were estimated by measuring the areas of the absorption curves at 254 with a Quantimet 720 image analysing computer.

Serology. Antisera were prepared as described by Bailey & Woods (1974) and immunodiffusion tests were done with the EDTA-phosphate agar described by Bailey & Woods (1977). Parts of individual bees were extracted for immunodiffusion tests in 0·05 ml or 0·1 ml of 0·85% saline + one or two drops of diethyl ether, according to the amount of tissue, by grinding them with small glass rods in small conical centrifuge tubes. This provided sufficient crude extract that could be pipetted to fill at least one well in the agar. Groups of 30 bees from laboratory experiments or field samples were extracted and the particles in them were sedimented, as described above, and resuspended in 1 ml of buffer for immunodiffusion tests.

Propagation. Adult bees and pupae were maintained in the laboratory and inoculated as described by Bailey & Woods (1974, 1977).

Histology. The thoracic musculature of live individuals from a colony naturally infected with CWP were used for sectioning. The thorax was bisected medially, one half placed in cold glutaraldehyde and the other extracted for immunodiffusion. The corresponding halves of those giving positive reactions with CWP antiserum were post-fixed in osmium tetroxide, dehydrated through a graded series of acetone and embedded in Spurr's low viscosity embedding medium (Glauert, 1975). Sections of muscle were stained with uranyl acetate for up to 1 h and with Reynold's lead citrate for 5 min and examined in a Phillips 201 electron microscope.

RESULTS

Chronic bee-paralysis virus associate

When CPV was extracted from bees found with chronic paralysis in the field, small peaks relative to those of CPV were seen sedimenting slowly in sucrose gradients (Fig. 1*a*). They were composed of isometric particles, about 17 nm in diam., that tended to form crystalline arrays (Fig. 2*b*). The particles had u.v. absorption spectra typical of nucleoproteins and were serologically unrelated to CPV. However, none of these has been seen in many extracts of healthy bees or in bees found severely infected in the field with bee virus X or black queen-cell virus (Bailey & Woods, 1974, 1977).

When groups of adult bees from seven different bee colonies were injected with the same

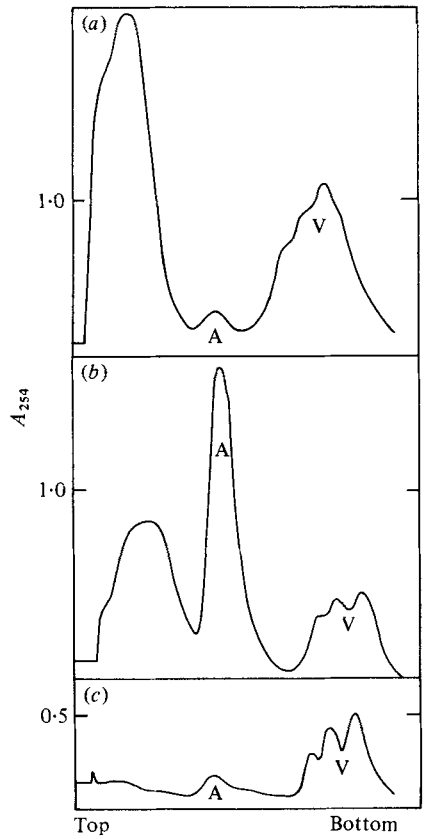

Fig. 1. Sucrose gradient analyses of extracts of tissues of bees suffering from chronic paralysis: (*a*) whole adult worker bees; (*b*) and (*c*) abdomens and heads, respectively, of a group of adult worker bees that produced unusually large amounts of CPVA. V = chronic paralysis virus, A = CPVA.

Table 1. *Physico-chemical properties of CPVA and CWP*

	Sedimentation coefficient	Buoyant density in CsCl (g/ml)	$A_{260}:A_{280}$	RNA (mol. wt.)	DNA	Proteins (mol. wt.)
CPVA	41 ± 1·3 (4)*	1·385 ± 0·003 (2)	1·24 ± 0·02 (2)	$1·0 \times 10^6$	ND†	15000
CWP	49 ± 0·6 (3)	1·383 ± 0·001 (3)	1·34 ± 0·03 (3)	$0·45 \times 10^6$	ND	19000

* Numbers in parentheses indicate the number of different preparations examined.
† Not detected (less than 5 % of the measured RNA).

partially purified preparation of CPVA + CPV the average amount (A_{254}) of CPVA relative to CPV obtained from six of the groups was 0·142 ± 0·23, as in Fig. 1 (*a*), but was 2·17 from one group (Fig. 1 *b*). Much more CPVA relative to CPV was produced in the abdomens than in the heads of bees (Fig. 1 *b*, *c*).

CPVA was not detected in pupae of worker bees after they had been killed by injection with crude preparations of CPV that contained CPVA, although the CPV had multiplied very much. However, pupae as well as adults of queen bees and some pupae of drone bees (male haploid individuals) that were similarly injected, produced large amounts of CPVA, about equal to the yield (A_{254}) of CPV. Purified CPVA did not multiply when injected alone into adult bees or pupae of worker or queen bees. However, experiments designed to test whether it could multiply only in the company of CPV were unsuccessful because it fre-

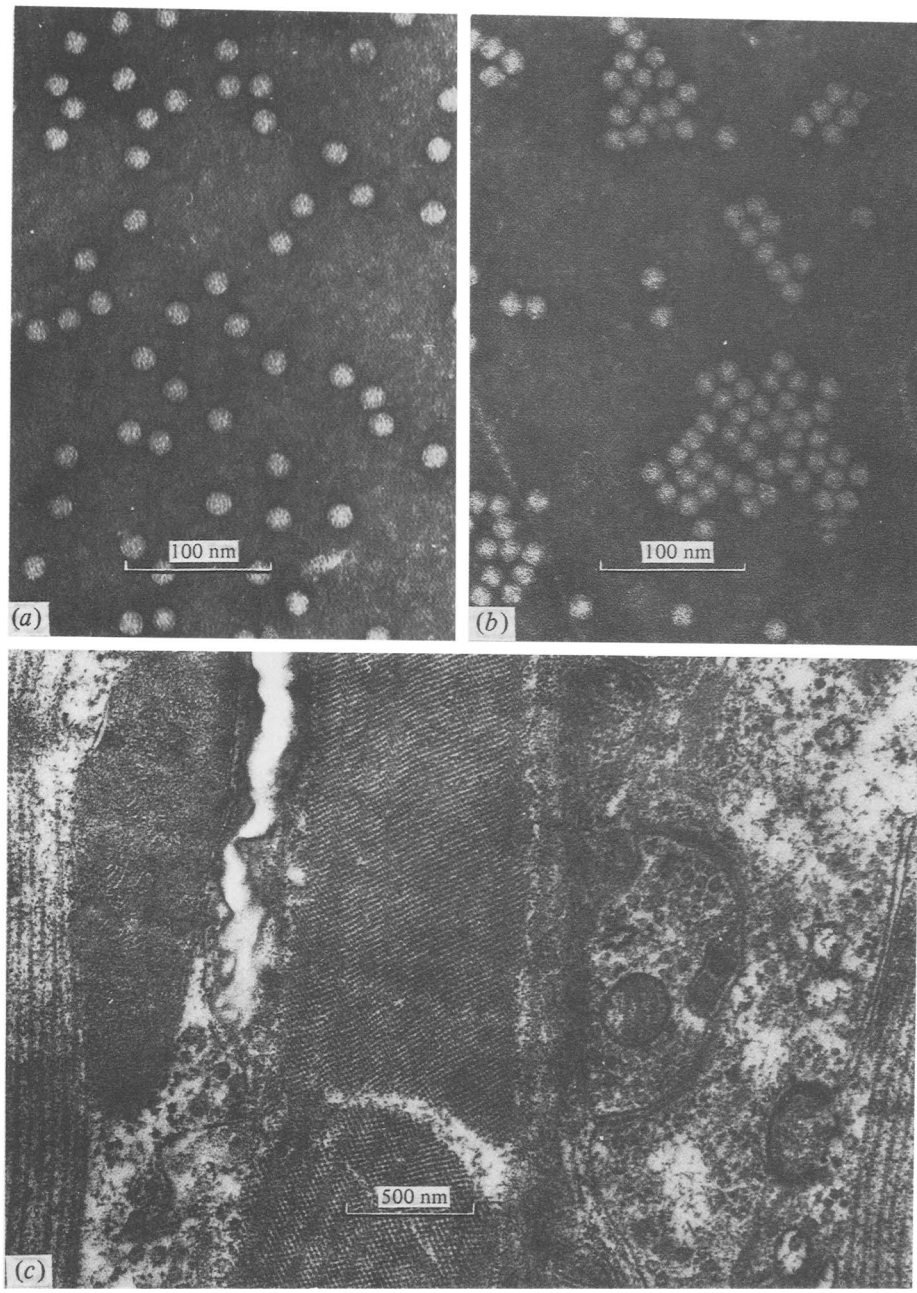

Fig. 2. Sodium phosphotungstate preparations of (*a*) CWP and (*b*) CPVA; and an ultrathin section (*c*) of thoracic muscle infected with CWP showing (left to right), muscle fibril, sarcosome, tracheole, crystal of particles, neuromuscular junction, sarcoplasm, muscle fibril.

quently multiplied in individuals injected with apparently purified CPV as well as in those injected with CPVA + purified CPV.

There was sufficient CPVA to be detected by immunodiffusion in the heads of some adult worker and drone bees and the heads and ovaries of adult queens that had died of chronic paralysis.

CPV was detected by immunodiffusion in the extracts of 27 out of 110 samples of dead adult bees collected in the field from beneath apparently normal bee colonies in the late winter of 1977. This group of 27 also contained 12 out of the 16 samples in which CPVA was detected. The association between CPV and CPVA in these tests is much greater than can be accounted for by chance (χ_c^2; $P < 0{\cdot}001$).

The physico-chemical properties of CPVA, derived from all the various sources described above, are given in Table 1. After purification by CsCl gradient centrifugation and Sephadex column chromatography, CPVA preparations gave only one major protein of mol. wt. 15000 and small amounts, probably of host protein, with mol. wt. of 60000 to 75000. The latter were probably of host origin because they were partially removed from the CPVA on the Sephadex column and migrated on acrylamide gels to the position of known host proteins.

Cloudy wing particle

Isometric particles the same size as CPVA were seen by electron microscopy (Fig. 2*a*) in extracts of adult bees that showed a marked loss of transparency of their wings and then died much earlier than had been anticipated during laboratory experiments with other viruses. The simultaneous occurrence of the disease in all of many groups of caged individuals, including uninoculated control groups, in one incubator but not in parallel groups in another, suggests that infection was airborne over a short range. No other viruses or virus-like particles were detected in the diseased individuals.

The yield of CWP from a standard number of bees was increased by at least 10-fold when 0·1 M-buffer was used instead of 0·01 M-buffer. Most of the particles were sedimented during the initial low speed centrifugation in 0·01 M-buffer but they could readily be recovered by re-extracting the pellet in more concentrated buffer. When this was done with a small vol. of 0·2 M-ammonium acetate at pH 5·0 and the extract then clarified again by low speed centrifugation, the particles re-suspended readily and were free from most contaminants. Re-extraction with concentrated buffers at higher pH values produced slightly more CWP, but the preparation then contained much amorphous material and was far less satisfactory for electron microscopy.

CWP did not multiply when fed to caged bees or when injected into adult bees or pupae. In some attempts to infect caged bees by spraying them or rubbing their thoraces with semi-purified preparations of CWP, all the inoculated individuals died after incubation periods of 10 to 14 days at 30 °C and contained much CWP. Other seemingly identical attempts and further experiments with different inocula, temperatures and sources and ages of bees failed completely.

There was sufficient CWP to give strong reactions in immunodiffusion tests in the heads, thoraces and thoracic musculature of individual bees that had been successfully infected in the laboratory and in bees found to be infected in the field. Extracts of the abdomens of the same individuals gave feeble or no reactions, but strong reactions were obtained with extracts of the abdomen as well as the head and thorax of the dead queen of a bee colony which had died and which contained much CWP but no other pathogens. There were no obvious histopathological differences between thoracic muscle infected with CWP and uninfected muscle, but particles in crystalline array, with centre to centre distances of about 17 nm, occurred between sarcolemmae of fibres of infected muscle (Fig. 2*c*). The physico-chemical properties of CWP are given in Table 1.

The mobility of the proteins and RNAs of CWP and CPVA were distinctly different. Shortage of purified material prevented further tests, but both RNAs appeared to be single-stranded because of their diffuse appearance on gels, unlike the sharp bands produced by double-stranded (ds) RNAs. The particles failed to react with antisera prepared against each other, but their buoyant densities in CsCl, determined simultaneously with minimal

quantities of CWP and CPVA both separately and mixed together, were identical. The relative areas of the u.v. absorption curves obtained in this analysis with CWP+CPVA: CWP:CPVA were 1:0·66:0·43, which agrees well with expectation, considering the likely errors involved; but there was no indication of asymmetry in the single peak produced by the mixture of approximately equal quantities of the two kinds of particle.

DISCUSSION

CPVA is associated, probably specifically, with CPV, but there is no experimental evidence that it depends on CPV for information to enable it to replicate. The variability in amounts of CPVA, relative to those of CPV, according to the kind of tissue or the source of bees in which both multiply, suggests that the multiplication of CPVA is greatly influenced by factors in the host. However, these factors may still be wholly determined by the paralysis virus, which may act differently in different tissues.

CPVA was apparently not always associated with CPV in some dead adult bees that were collected in the field but these failures to detect one particle in the presence of the other could have been due to the insensitivity of the method of detection and to the variations in the proportion of CPVA to CPV. CPVA multiplied in some individuals that were injected with purified CPV, either because the virus was not completely free of CPVA, or because this was already in the insects, which are commonly and inapparently infected with a variety of viruses, including CPV (Bailey, 1976).

Another particle physically similar to CPVA has been reported to be associated with a virus of the emperor gum moth, *Antheraea eucalypti* (Bellett *et al.* 1973; Reinganum, 1975). By contrast with this particle and with CPVA, the cloudy wing particle seems to multiply independently of any other virus. This is not without precedent for a virus with such an unusually small particle in insects since the similarly sized crystalline array virus of the grasshopper, *Melanoplus bivittatus* (Jutila *et al.* 1970) has not been associated with larger virus particles. The small size of these particles makes their identification uncertain in ultrathin tissue sections except when they form crystalline arrays, and those of CWP were seen only in spaces between sarcolemmae. However, since CWP seems to be transmitted through the air, initial infection and replication may well be in the tracheal matrix, as reported for the grasshopper virus (Henry & Oma, 1973), and especially among the muscle fibrils of the thorax, where the main inhalatory spiracles occur.

Although some infectivity tests with semi-purified CWP were successful, the possibility of other factors that are essential for infection or pathogenesis cannot be excluded and their absence may explain the failures we experienced with some preparations. Furthermore, cloudy wing symptoms do not seem to be invariably or specifically associated with severe infection by CWP. We have failed to detect the particles in some bees that were dead and had cloudy wings when collected in the field and, in contrast, have detected many of the particles in others not showing any external signs of disease.

In view of the similar sizes of CWP and CPVA and the apparently large difference between the mol. wt. of their RNAs, their indistinguishable buoyant densities seem unexpected. The discrepancy cannot simply be resolved by postulating two RNAs, each of mol. wt. $0{\cdot}45 \times 10^6$, in CWP because of other properties of the particle. For example, its size and sedimentation coefficient and the mol. wt. of its protein and nucleic acid closely resemble those of the satellite particle of tobacco necrosis virus (Kassanis, 1970), which has a single RNA of mol. wt. $0{\cdot}4 \times 10^6$. By contrast, CPVA appears to have an RNA of an unusually high mol. wt. to be compatible with some of its other properties, especially its low buoyant density and $A_{260}:A_{280}$ ratio. It might have other unusual and compensating characteristics such as lipid or carbohydrate, or possibly its RNA aggregated in the gels. These did not contain urea or

formamide, although the RNAs were heated to 60 °C in 1 % SDS and 4 M-urea immediately before loading.

We now know that both CPVA and CWP are very common among bees in Britain and we have detected CWP in samples of bees from Egypt and Australia. Moreover, as we have found CWP associated with severe dwindling and ultimate disappearance of bee colonies in Britain, it must be considered as another of the many agents, mostly viruses, that attack bees and that may cause or contribute towards many unexplained losses.

We thank Dr D. A. Govier, Rothamsted Experimental Station, for preparing the antisera.

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(*Received* 21 *May* 1979)