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Two More Small RNA Viruses from Honey Bees and Further Observations on Sacbrood and Acute Bee-Paralysis Viruses

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SUMMARY

Black queen-cell virus was isolated from dead prepupae and pupae of queens and workers of *Apis mellifera* found locally in the field. Kashmir bee virus was isolated from individuals of *Apis mellifera* that had died in the laboratory after they had been inoculated with some preparations from *Apis cerana*. Both viruses have isometric particles about 30 nm in diameter, contain RNA, and are unrelated to each other or to any known bee virus. Black queen-cell virus particles sediment at 151S and have a buoyant density in CsCl of 1·345 g/ml; Kashmir bee virus particles sediment at 171 to 173S and have a buoyant density in CsCl of 1·371 g/ml. Serological evidence indicates that black queen-cell virus is common in Britain and occurs in the U.S.A. The studies involved acute bee-paralysis and sacbrood viruses and led to re-determination of the buoyant densities of these as 1·380 and 1·358 g/ml respectively.

INTRODUCTION

Seven viruses from bees have previously been described. Five of them have small isometric particles containing RNA (Bailey, Gibbs & Woods, 1963, 1964; Bailey & Woods, 1974), one has an isometric particle also containing RNA (Bailey *et al.* 1963) and one is an iridovirus (Bailey, Ball & Woods, 1975). Recently we have isolated two more viruses with isometric particles, one from *Apis cerana*, the eastern hive-bee and one from *Apis mellifera*, the European honey bee. During this work we were led to make further observations on sacbrood and acute bee-paralysis viruses.

METHODS

Methods of virus propagation, purification bioassay and serology were mostly as described by Bailey & Woods (1974). Additional methods and modifications were as follows.

Bioassay and virus propagation. Young (white-eyed) bee pupae were sometimes more convenient than adult bees for propagating viruses and for bioassay. The pupae were collected from bee combs, injected through a dorsal intersegmental membrane with 1 μl of test preparations, and then kept in small dishes placed on filter paper soaked in 12% (v/v) glycerol containing 10 mg/100 ml Thiomersal (BDH Chemicals Ltd., Poole), within a Petri dish. All, or nearly all, control pupae, which were injected with virus-free solutions, developed normally at 30 or 35 °C with no further attention, and they metamorphosed into adult bees. We have found that bee pupae are very suitable for propagating all the bee viruses, except bee virus X (Bailey & Woods, 1974), and for culturing several other insect viruses, e.g. *Tipula*, *Sericesthis* and *Chilo* iridescent viruses, and *Drosophila* C virus (Jousset *et al.* 1972).

Virus purification. Bees were extracted in 4 vol. of 0·01 M-potassium phosphate buffer at pH 7·0 + 0·2% sodium diethylthiocarbamate + 1 vol. diethyl ether. The mixture was emulsified with CCl_4 and cleared by centrifuging at 8000 *g* for 10 min.

The clarified extracts of pupae contained very many particles that seemed to be aggregates of varying numbers of almost spherical units, each about 20 nm across. The range of sedimentation coefficients ($s_{20,w}$) of these aggregates overlapped those of all the viruses investigated, preventing the purification of the viruses by differential centrifugation or in sucrose gradients. However, keeping the extracts for a few days at 4 °C and centrifuging them for 10 min at 8000 *g* each day usually removed the aggregates. Freezing the extracts was not helpful and sedimented aggregates quickly redissolved at 20 °C. Recently we found that acidifying the crude suspension to pH 5·0 with 0·1 M-acetate buffer precipitated the aggregates at once at 20 °C, without affecting black queen-cell virus (see below), sacbrood virus or acute bee-paralysis virus. Preparations could then be cleared by low speed centrifugation for a few minutes. Citrate–phosphate buffer at pH 5·0 precipitated the aggregates less effectively than acetate buffer.

Sedimentation coefficients and buoyant densities in CsCl were determined in a Spinco Model E analytical ultracentrifuge (Bailey & Woods, 1974) or, under similar conditions, in an MSE Centriscan centrifuge. The two centrifuges gave identical results.

U.v. absorption spectra of purified virus preparations were determined in a Unicam quartz spectrophotometer Model SP 500, checked for accuracy with a holmium filter.

Serology. The most satisfactory agar for immunodiffusion contained 0·05 M-potassium phosphate buffer (pH 7·0) + 0·005 M-EDTA + 0·2% sodium azide. This agar has also proved most suitable for all other bee viruses except Arkansas bee virus.

RESULTS

Black queen-cell virus

During July 1975 many prepupae and a few pupae were found dead in the cells in which queens were being reared, by standard methods, in local colonies of bees (*Apis mellifera*). The pupae were partially decomposed and darkened and their cell walls appeared almost black in patches. Similar examples were found in April and May of the following year, including dead pupae of worker bees. Electron microscopy of extracts of the dead specimens showed at least 10^{12} isometric particles, 30 nm in diam., per individual (Fig. 1*a*). The symptoms closely resembled those of sacbrood (Bailey *et al.* 1964) but no reactions were obtained with the dilutions of sacbrood virus antiserum we used for diagnosis.

The particles, which we call black queen-cell virus, had u.v. absorption spectra typical of nucleoproteins and contained RNA but not DNA as indicated by positive orcinol and negative diphenylamine reactions. They multiplied when injected into pupae, and these each contained at least 10^{12} particles after a few days and failed to develop. However, infectivity tests on larval and adult bees, similar to those done successfully with sacbrood virus (Bailey, 1969), usually failed; and by contrast with sacbrood virus (Bailey & Fernando, 1972) black queen-cell virus did not multiply in the brains of injected drones sufficiently to be detected by immunodiffusion.

The differences in behaviour between the viruses in these and other bioassays were possibly of degree rather than kind. For example, black queen-cell virus was morphologically unstable below pH 4·0, like sacbrood virus (Newman *et al.* 1973), but about 10% of the particles in preparations of black queen-cell virus remained intact at pH 3·0 after 20 min whereas all sacbrood virus particles disintegrated. Furthermore, an antiserum prepared

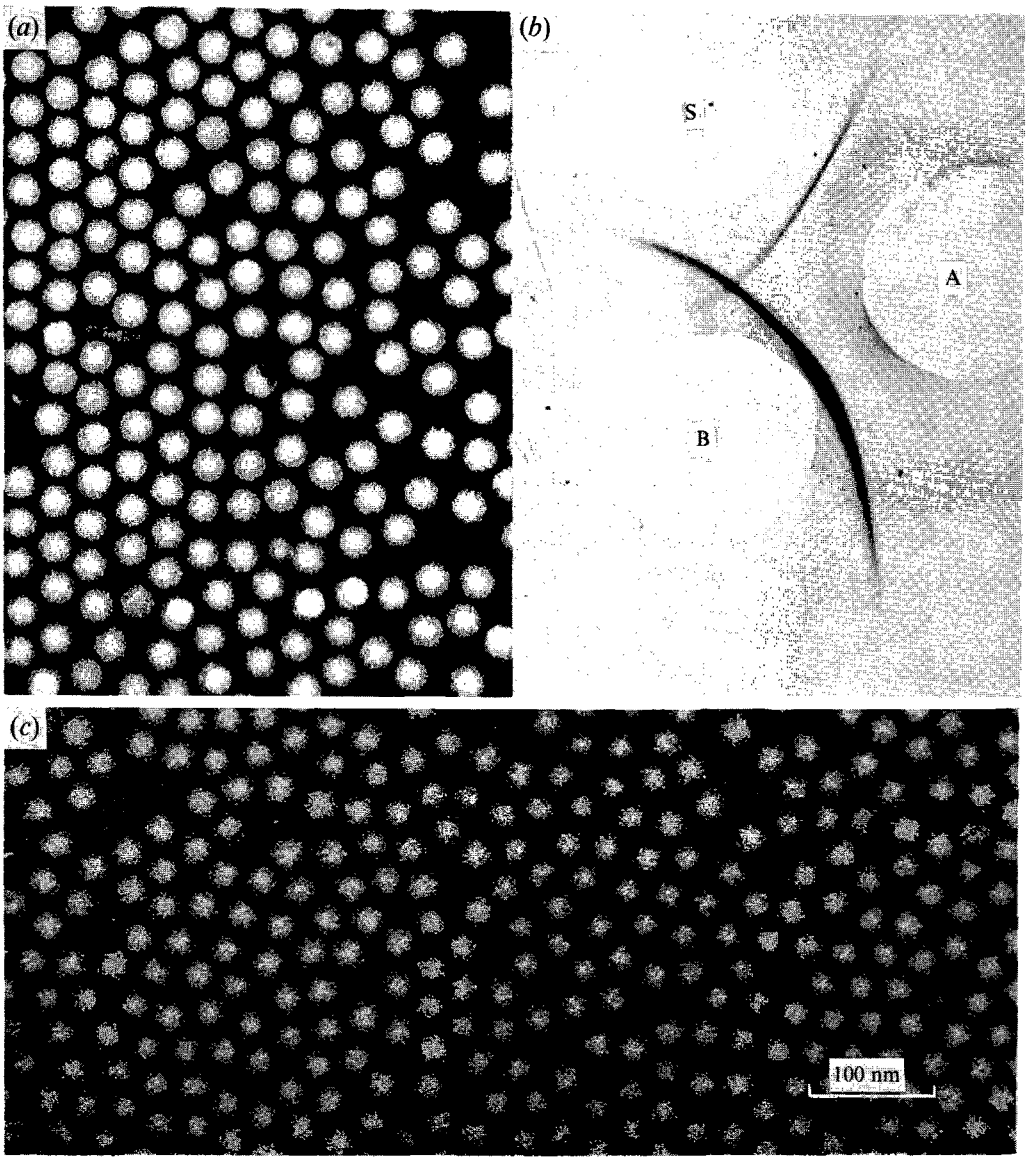

Fig. 1. (*a*) Black queen-cell virus. (*b*) Immunodiffusion test against black queen-cell virus (B) and sacbrood virus (S). The antiserum (A), diluted 1/16, was prepared against black queen-cell virus, against which it had a titre of 1/512. (*c*) Kashmir bee virus.

against black queen-cell virus, with an homologous titre of 1/512, had a titre against sacbrood virus of 1/32; and two antisera prepared several years previously against sacbrood virus had low titres against black queen-cell virus. Nevertheless, immunodiffusion tests against purified preparations of sacbrood and black queen-cell virus with these antisera gave lines of precipitation that crossed sharply, with no sign of confluency, spurring or loss of intensity after crossing (Fig. 1*b*). This suggested that antisera had been prepared against a mixture of two unrelated viruses. However, the sedimentation coefficient of black queen-cell virus, determined with four different preparations was only 151S (standard error 0·8),

Table 1. *Titres of some bee virus antisera against black queen-cell virus*

Antiserum	Date prepared	Homologous titre	Titre against black queen-cell virus
Acute bee-paralysis virus	(1) Jan. 1969*	1/128	1/128
	(2) Nov. 1975	1/512	No reaction
Arkansas bee virus	(1) Sept. 1972†	1/64	1/8
	(2) Dec. 1975	1/512	No reaction
Bee virus X	Mar. 1973	1/256	No reaction
Sacbrood virus	(1) July 1963	1/64	1/4
	(2) Nov. 1969	1/256	1/4
Slow paralysis virus	Dec. 1973	1/64	1/8

* Prepared from virus only semi-purified by differential centrifugation.
† Prepared in the U.S.A.

and there was no sign in the Schlieren diagram of a second peak caused by sacbrood virus, which has a sedimentation coefficient of 157S (Newman *et al.* 1973) to 160S (Bailey *et al.* 1964). Moreover, buoyant density determinations with two separate preparations of black queen-cell virus, which were subsequently pooled and used to make the antiserum, showed a major fraction with a buoyant density at pH 7·0 of 1·345 g/ml (standard error 0·002) and a minor one, indicated by u.v. absorption only, with a buoyant density of 1·357 g/ml (standard error 0·002); there was no sign of a fraction with a buoyant density of 1·33 g/ml, the value reported for sacbrood virus by Newman *et al.* (1973). However, two further preparations of black queen-cell virus, each with a buoyant density of 1·344 g/ml (standard error 0·002) at pH 7·0 (1·343 g/ml at pH 9·0) lacked the minor denser fraction found in the previous two. This, together with the serological evidence, led us to re-examine the buoyant density of sacbrood virus. A purified preparation of this virus, which had a sedimentation coefficient of 159S, had a buoyant density of 1·358 g/ml. Therefore, the black queen-cell virus preparations showing two fractions in the buoyant density determinations were probably contaminated with a relatively small amount of sacbrood virus, which has a considerably higher density, at least under our conditions, than that reported by Newman *et al.* (1973). The source of the contamination is unknown but it probably came from the apparently healthy pupae used for the cultivation of black queen-cell virus, as sacbrood virus commonly occurs in seemingly healthy bee colonies (Bailey, 1967).

The results of further investigations of the titres of bee virus antisera against black queen-cell virus are shown in Table 1. Cross-reactions were common, particularly with antisera prepared before black queen-cell virus had been recognized. Immunodiffusion tests indicated no relationship between this virus and any of those against which the antisera had been prepared.

Kashmir bee virus

During investigations with *Apis* iridescent virus (Bailey *et al.* 1975), virus-like particles about 30 nm in diam. were isolated from adult honey bees (*Apis mellifera*) that had ingested suspensions of the original iridescent virus prepared from *A. cerana*. Similar particles (Fig. 1 *c*) were isolated from adult bees that died after they had been injected with preparations of *Apis* iridescent virus that had been partially purified by three cycles of centrifugation at 1000 ***g*** for 30 min and 15000 ***g*** for 10 min. This suggested that the small particles had been adsorbed to the iridescent virus particles, as did our observation by electron microscopy of

many similar particles among other preparations of iridescent virus particles that had been purified by five cycles of centrifugation at 1000 ***g*** and 15000 ***g***.

Adult bees died within 6 days at 30 or 35 °C when injected with the particles, which we call Kashmir bee virus, but they seemed unaffected when they ingested them. Up to 10^{13} particles were extracted from injected individuals, and most virus accumulated in individual adult bees or pupae injected with the highest infective dilutions, as occurs with Arkansas bee virus (Bailey & Woods, 1974).

The particles had u.v. absorption spectra typical of nucleoproteins and contained RNA but not DNA according to positive orcinol and negative diphenylamine reactions. Serological tests using antisera to other bee viruses indicated no relationship between the 30 nm particles and any of these viruses. EDTA agar was satisfactory for immunodiffusion tests but the particles aggregated spontaneously in agar containing 0·85% sodium chloride, in a manner resembling that of slow bee-paralysis virus (Bailey & Woods, 1974).

Concentrated preparations of Kashmir bee virus crystallized in 0·85% saline. The crystals sedimented readily at 8000 ***g*** and dissolved when suspended in water or phosphate buffer. Sufficient virus was readily purified in sucrose gradients to obtain accurate dry weights and 1 mg/ml had an extinction/cm at 260 nm of 5·0.

The sedimentation coefficient of Kashmir bee virus was 173S in phosphate buffer and 171S in 0·1 M-KCl. The buoyant density in CsCl at pH 7·0 of four separate preparations was 1·371 g/ml (standard error 0·002). Only one component was seen in these studies but an antiserum prepared against Kashmir bee virus, with an homologous titre of 1/256, had a titre against acute bee-paralysis virus of 1/64, indicating a possible relationship between the two viruses. However, immunodiffusion tests in which the antiserum was tested against purified preparations of the two viruses in adjacent wells gave crossed lines of precipitation with no indication of relationship; and an antiserum prepared against acute bee-paralysis virus did not react with Kashmir bee virus.

Higher pH values increase the buoyant density of acute bee-paralysis virus, e.g. 1·34 g/ml at pH 7·0 to 1·42 g/ml at pH 9·0 (Newman *et al.* 1973). When similar tests were made on Kashmir bee virus, the preparation resolved into two components at pH 9·0. The major component had a density of 1·384 g/ml and the minor one, which was seen only by u.v. absorption, had a density of 1·420 g/ml. This, together with the serology, led us to suspect that the preparation was contaminated with acute bee-paralysis virus, although there was no sign of a component at pH 7·0 with a density of 1·34 g/ml. However, three separate preparations of acute bee-paralysis virus, two cultured in adult bees and one in pupae, had a buoyant density of 1·380 g/ml (standard error 0·003), and this increased to 1·420 g/ml at pH 9·0. Therefore, the preparations of Kashmir bee virus had probably become contaminated with a relatively small amount of acute bee-paralysis virus. This, like sacbrood virus, has a considerably higher buoyant density in CsCl at pH 7·0, under our conditions, than that reported by Newman *et al.* (1973), and at this pH its density is not sufficiently different from that of Kashmir bee virus to enable the viruses to be separated readily by isopycnic centrifugation.

Four separate preparations of acute bee-paralysis virus, three from adult bees and one from pupae, had an average sedimentation coefficient of 163S (standard error 0·4), slightly higher than that of 160S found by Bailey *et al.* (1963) and Newman *et al.* (1973), but they were all serologically indistinguishable from each other and from the earlier acute bee-paralysis virus preparations used to make antisera. When purified, acute bee-paralysis virus aggregated spontaneously in agar containing 0·85% saline but it was stable in the EDTA agar used for immunodiffusion tests, resembling slow paralysis and Kashmir bee viruses in these respects.

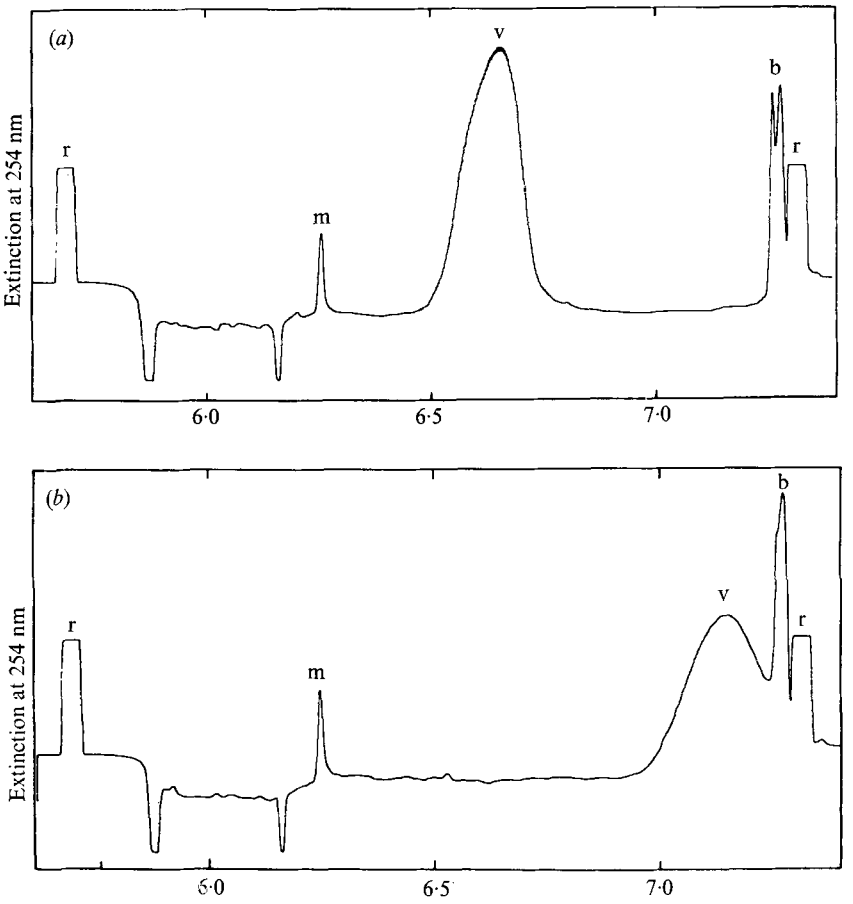

Fig. 2. U.v. scan of acute bee-paralysis virus equilibrated at 25 °C in CsCl, buffered with 0·03 M-borate, after 17 h at 44000 rev/min in an MSE Centriscan, (*a*) at pH 7·0, original density 1·395 g/ml and (*b*) at pH 9·0, original density 1·397 g/ml. m = meniscus, v = virus, b = bottom of cell, r = reference marks.

Table 2. *Characteristics of bee viruses with isometric 30 nm particles*

Virus	Sedimentation coefficient	Buoyant density in CsCl (g/ml) pH 7·0	pH 9·0	E_{260}/E_{280}
Acute bee-paralysis	163	1·38	1·42–1·43	1·79
Arkansas bee	128	1·37		1·37
Black queen-cell	151	1·34	1·34	1·71
Kashmir bee	171–173	1·37	1·38	1·60
Sacbrood	159	1·36		1·82
Slow bee-paralysis	172–178	1·37*		1·50

* Misquoted as 1·35 by Bailey & Woods (1974).

Most of the buoyant density determinations described above, including those of black queen-cell virus were done in 0·005 M-phosphate buffered CsCl, but 0·03 M-phosphate, used to study the effects of changing pH, gave similar and consistent results. Acute bee-paralysis virus in tris-buffered CsCl seemed unstable during isopycnic centrifugation, becoming dispersed between densities of 1·380 and 1·41 g/ml at pH 7·0 and undetectable at pH 9·0. By contrast, even after 14 days in 0·03 M-phosphate-buffered CsCl at pH 7·0, the density of the virus changed only slightly from 1·380 to about 1·390 g/ml. Results with acute bee-paralysis virus in borate buffer (0·03 M) were very similar to those in phosphate, with buoyant densities of 1·381 g/ml at pH 7·0 and 1·435 g/ml at pH 9·0 (Fig. 2).

Table 2 gives a revised list of some of the physical properties of the known bee viruses with isometric 30 nm particles.

DISCUSSION

The identification of the small isometric RNA viruses of bees is handicapped by the lack of alternative hosts, in which they might be propagated specifically, by their common occurrence in seemingly healthy bee colonies, and by the difficulties of separating them from each other by physical means. Therefore it would be rash to assume that all have been found, especially as black queen-cell virus is associated with manifest disease in nature and has, nevertheless, escaped detection for so long. The bee viruses illustrate the possibility that certain similar viruses identified in some other insects will prove to be groups of unrelated types that are equally difficult to distinguish and separate by physical means.

The prevalence of reactions against black queen-cell virus by antisera prepared against different bee viruses on widely separate occasions suggest that black queen-cell virus occurs commonly in Britain. Current surveys are now confirming this (L. Bailey, unpublished observations). Moreover, the reaction of the Arkansas bee virus antiserum that was prepared in Arkansas in 1972 (see Table 1) shows that black queen-cell virus occurs in the U.S.A. The virus may well be common in *Apis mellifera* in many other countries, but its effects are not easily recognized in the field and it is not readily cultivated except by injection into bee pupae. This almost certainly does not reflect the method by which it is propagated in nature.

The evidence that Kashmir bee virus came from *Apis cerana* is circumstantial. However, it probably does not exist in Britain, or it would have been noticed previously in experiments at Rothamsted, since it multiplies so abundantly and quickly. Whether it is invariably associated with *Apis* iridescent virus in nature is uncertain, but it has multiplied in bees injected with extracts of individuals of *A. cerana* that were apparently free of the iridescent virus and were received from elsewhere in India.

The discrepancies between the buoyant densities in CsCl at pH 7·0 of sacbrood and acute paralysis viruses when determined by Newman *et al.* (1973) and by us are not readily explained. Possibly the observed densities of both these viruses increase with the period of centrifugation, as Rowlands, Sangar & Brown (1971) found with certain viruses, such as foot-and-mouth disease virus. We observed the densities after equilibration at 44000 rev/min for 17 h, whereas Newman *et al.* (1973) centrifuged their preparations in pre-formed gradients for only 6 h. However, Rowlands *et al.* (1971) found that animal picornaviruses in the lower density range (about 1·34 g/ml) were unaffected by the period of centrifuging. Therefore, if acute bee-paralysis and sacbrood viruses are indeed affected by the period of centrifugation, it will help to differentiate them further from the mammalian picornaviruses. Another possible reason for the higher densities we observed for these two bee viruses is that we purified them by a method different from that used by Newman *et al.* (1973) and our

method may be better for these particular viruses since we obtained relatively high E_{260}/E_{280} ratios. The similar density observed by Newman *et al.* (1973) to those we found for acute bee-paralysis virus at pH 9·0 may mean that the virus particles in their preparations lost adsorbed contaminants at the higher pH. More exacting studies, such as those done by Rowlands *et al.* (1971) are needed to measure the effects of different buffers and of periods of centrifugation on the buoyant densities of the viruses of bees and of other insects, although the methods we used were reliable and adequate for our purpose.

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