

Three Previously Undescribed Viruses from the Honey Bee

By L. BAILEY AND R. D. WOODS

Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, U.K.

(Accepted 20 June 1974)

SUMMARY

Arkansas bee virus, bee virus X and slow paralysis virus, isolated from adult honey bees, have isometric particles, contain RNA and are serologically unrelated to each other or to the other known bee viruses. Arkansas bee virus particles are 30 nm in diam. sediment at 128S, have a buoyant density in CsCl of 1.37 g/ml and kill bees injected with them in about 3 weeks. Bee virus X particles are 35 nm in diam., sediment at 187S, have a buoyant density of 1.36 g/ml, multiply when fed to young bees kept at 30 °C but not at 35 °C nor when injected, and have not by themselves been associated with symptoms or mortality, although they killed bees when injected in combination with sacbrood virus. Slow paralysis virus particles are 30 nm in diam., sediment at about 176S, have a buoyant density of 1.35 g/ml and kill bees injected with them in about 12 days.

INTRODUCTION

The viruses that have previously been isolated from honey bees are chronic and acute bee-paralysis viruses (Bailey, Gibbs & Woods, 1962) and sacbrood virus (Bailey, Gibbs & Woods, 1963). Acute bee-paralysis and sacbrood viruses are picornaviruses (Newman *et al.* 1973); chronic bee-paralysis, also containing RNA, has asymmetric virus particles that vary considerably in size and shape. The paralysis viruses kill adult honey bees and sacbrood virus kills their larvae, but all these viruses can persist in apparently healthy adult bees (Bailey, 1967; Bailey & Fernando, 1972) and occur in the pollen loads of inapparently infected foraging bees (Bailey, 1971). These inapparent infections, which are common, at least in Britain (Bailey, 1967; Bailey & Gibbs, 1964) can be detected by injecting bacteria-free extracts of apparently healthy adult bees or of their pollen loads into similar individuals. Any of the three viruses in the extracts will then multiply systemically in the injected bees, sufficiently to be readily identified by serological tests. Using this method for detecting inapparent infections we have isolated a previously undescribed virus from honey bees in Arkansas, U.S.A., and in consequence of work with this virus, found two more viruses in bees in Britain.

METHODS

Bioassay. Adult bees were anaesthetized with CO₂ and each injected by means of a sterile microsyringe with 1 µl of test preparations through a dorsal abdominal intersegmental membrane. In some tests unanaesthetized bees were fed with test preparations either by placing 2 µl of the liquid directly between their mandibles with a microsyringe, while they were held by their wings, or by wetting, as evenly as possible, 20 individuals confined in a cage of 30 cm³, by dripping 0.5 ml of a preparation on them. Bees readily ingested fluids that

were sweetened with 10 % honey. They were then maintained in cages ($25 \times 25 \times 100$ mm), 20 or 30 individuals per cage and supplied with concentrated sucrose solution and water in gravity feeders. Newly emerged bees, obtained by incubating mature pupae at 35°C , were also supplied with pollen (Bailey, 1969).

Inocula. Bees, or pollen loads knocked from the legs of returning foragers by means of a wire mesh through which the bees had to pass into their colony, were triturated in a 4:1 mixture of 0.01 M-potassium phosphate buffer at pH 7.0 (PB) and ethyl ether. The mixture was then emulsified with 1 vol. of carbon tetrachloride, coarsely filtered and cleared at 8000 g for 10 min. Ten bees or 1 g of pollen were extracted per ml. of PB.

Virus purification. Bees were extracted in 4 vol. PB + 0.02 % sodium diethyldithiocarbamate (to prevent melanization) + 1 vol. ethyl ether. The mixture was emulsified with CCl_4 and cleared, as for inocula, and the cleared fluid centrifuged at 75000 g for 3 h or 100000 g for $1\frac{1}{2}$ h. Later (see *Slow paralysis virus* below) it was found advantageous to add about 3 ml of Triton X-100 (Rohm and Haas Co.) to 100 ml of clarified extract before the high-speed sedimentation, which then produced smaller pellets with less debris than from untreated extracts.

The pellets were resuspended in PB, cleared at 8000 g for 10 min and then centrifuged at 45000 g for 3 h down linear 10 to 40 % (w/v) sucrose gradients in PB. Fractions were collected from the top of the tube, by displacement from beneath with 50 % sucrose, and assayed for E_{254} , by means of an ISCO density gradient fractionator.

Sedimentation coefficients ($s_{20,w}$) were determined in a Spinco model E analytical ultracentrifuge by the graphical method described by Markham (1960). Buoyant densities in caesium chloride were determined, also in the model E centrifuge, after equilibration at 44000 rev/min for 17 h at 20°C . Virus preparations were negatively stained with neutral sodium phosphotungstate (PTA) and examined and photographed in a Siemens Elmiskop 1 electron microscope.

Serology. Antisera were prepared in rabbits against purified virus preparations by giving one intramuscular injection of 1 mg of virus, or more injections at about weekly intervals with about 0.1 mg in 1 ml of PB emulsified with an equal vol. of Freund's complete adjuvant. Sera, which gave the highest titres in immunodiffusion tests about 6 weeks after the final injection, were stored at -20°C .

Immunodiffusion tests as described by Mansi (1958) were done with purified virus or crude extracts made by grinding the head or abdomen of a bee in 0.05 ml of 0.85 % saline + 1 drop of ethyl ether in a small conical tube. The agar contained 0.04 M-sodium borate buffer, pH 7.0, + 0.85 % NaCl + 0.02 % sodium azide or 0.02 M-potassium phosphate buffer, pH 7.0, + 0.01 % KCl + 0.02 % sodium azide.

RESULTS

Arkansas bee virus

Many bees injected in Arkansas (U.S.A.) with extracts of locally gathered pollen loads died between 15 and 25 days later, whereas few or no bees died when injected with PB. Pellets obtained by high-speed sedimentation of extracts of the killed bees contained many isometric particles about 30 nm in diam. (Fig. 1*a*). When an extract of 15 bees dying following infection was pelleted and resuspended in 1 ml there was sufficient virus to give a well-defined peak in the analytical centrifuge by Schlieren optics. The sedimentation coefficient was 120S.

When further purified in sucrose gradients, a major component containing the virus-like

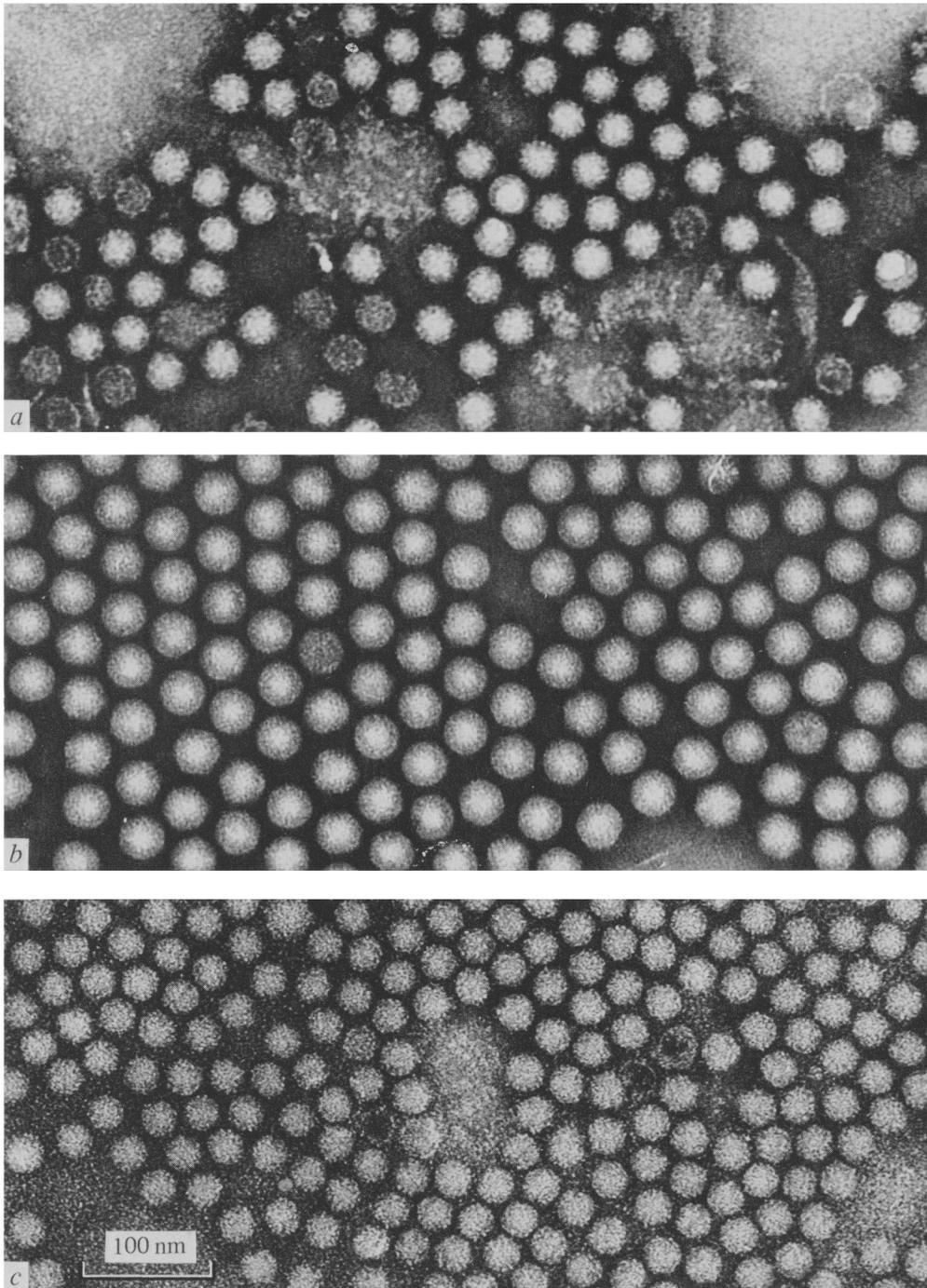


Fig. 1. Sodium phosphotungstate preparations of (a) Arkansas bee virus, (b) bee virus X and (c) slow paralysis virus.

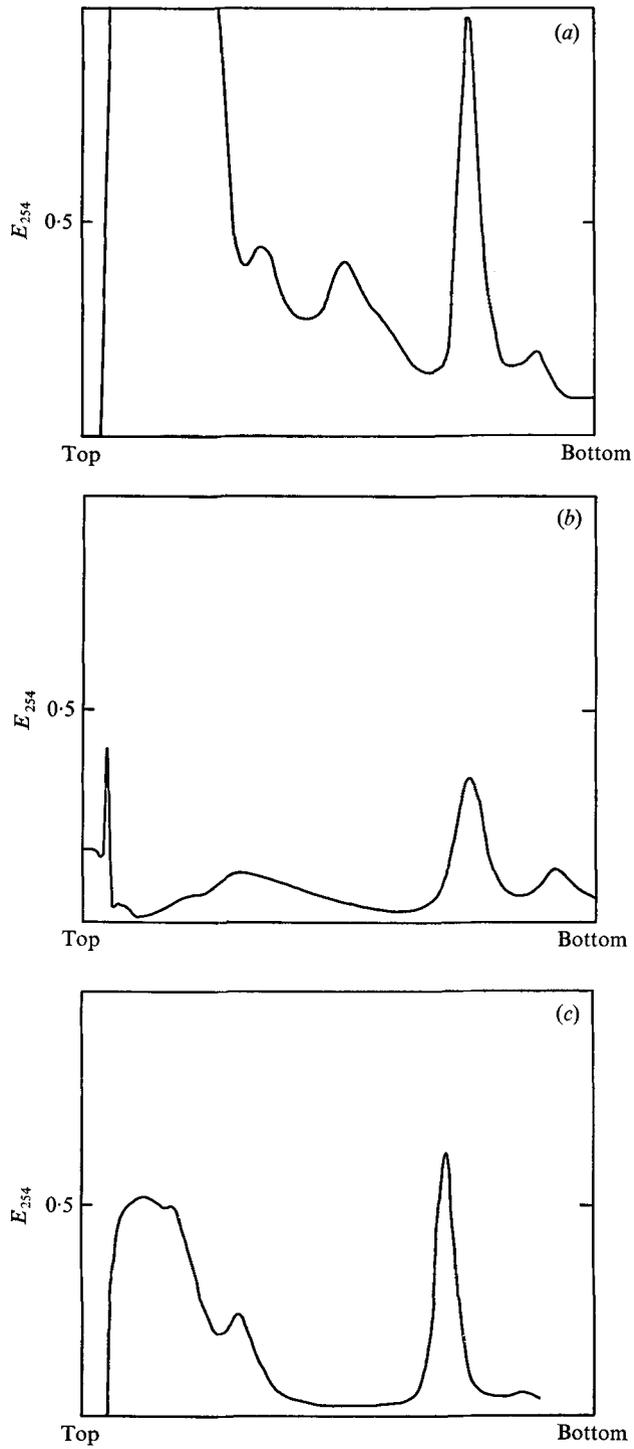


Fig. 2. Sucrose gradient analysis of bee extracts containing Arkansas bee virus (a) sedimented once, (b) sedimented twice, (c) sedimented twice and stored at 4 °C for 7 days before analysis.

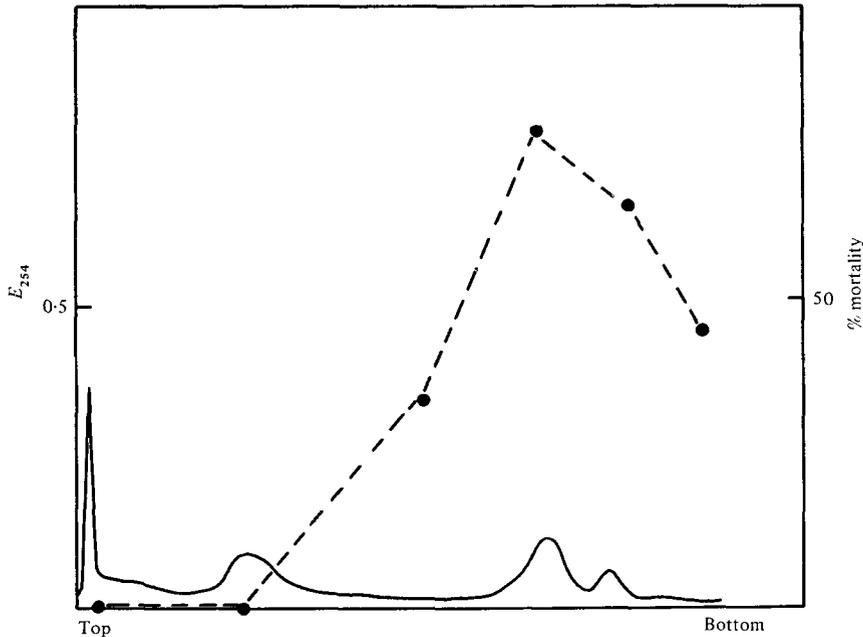


Fig. 3. Sucrose gradient analysis of semi-purified Arkansas bee virus showing distribution of E_{254} , and infectivity (● --- ●) of 1 ml fractions.

particles was typically preceded by a minor component (Fig. 2 and 3). After dialysis, the sedimentation coefficients of these components were 128S and 170S, respectively. Electron microscopy showed that both components contained apparently identical particles. The proportions of these two components varied but the minor component was increased by pelleting the virus and decreased by storing virus suspensions (Fig. 2). The minor component never appeared in the analytical ultracentrifuge by Schlieren optics when resuspended pellets of crude extracts were examined. Both components gave u.v. extinction curves typical of nucleoproteins (Fig. 5a) and significantly more infectivity was associated with them (Fig. 3) than with the slower sedimenting components, which were composed of amorphous material.

An extract of the head of one bee killed by the virus gave a strong single precipitin line in immuno-diffusion tests against suitable diluted antiserum using borate or phosphate buffer. Sometimes a second line formed very close to the antigen well. The preparations did not react with antisera prepared against any of the other bee viruses. Also, unlike any previously described bee virus, the Arkansas bee virus did not react against its homologous antiserum in agar containing buffer + 0.05 M-EDTA although the virus particles appeared unchanged after 24 h in 0.05 M-EDTA, when examined by electron microscopy. The effect of EDTA was not neutralized by mixing the virus with normal rabbit serum (*cf. Slow paralysis virus* below).

Extracts of bees killed by Arkansas bee virus seemed to contain few lethal doses by injection, but it became evident that virus multiplied in bees injected with high dilutions of these extracts, even though the infected bees then lived about as long as uninfected individuals. The least infective dose was found by injecting bees with a series of dilutions, extracting small groups of live individuals from each treatment after 18 days, clearing and centrifuging

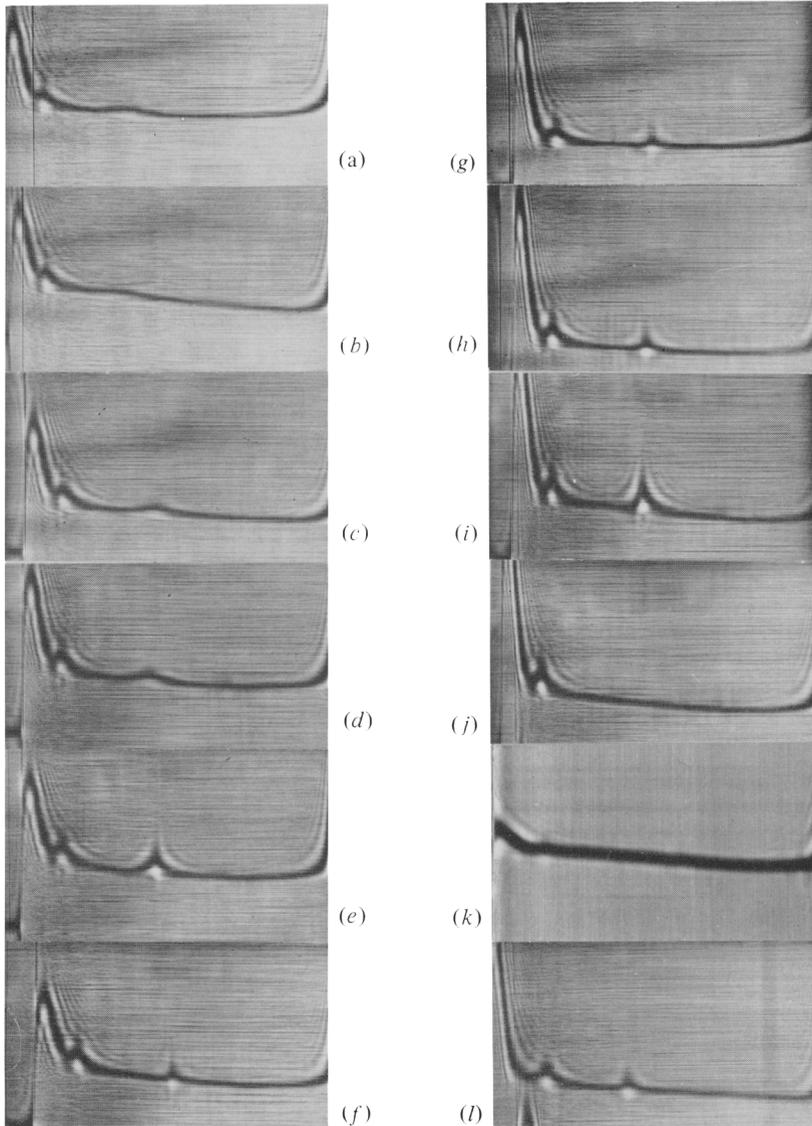


Fig. 4. Schlieren diagrams of sedimentation analysis of extracts of bees that had been injected 17 to 18 days previously with extracts of bees killed by Arkansas bee virus. Photographed after 12 min at 34 000 rev/min and 20 °C; bar angle 45°. Extracts were each of: (Expt. 1) 20 live bees, each injected with the equivalent of (a) 10^{-4} , or (b)–(f) tenfold dilutions respectively to 10^{-9} , of 1 killed bee; (Expt. 2) 15 live bees, each injected with the equivalent of (g) 10^{-8} , or (h)–(j) 100-fold dilutions respectively to 10^{-11} , of 1 killed bee; (Expt. 3) 20 bees killed by injection each with the equivalent of (k) 10^{-3} or (l) 10^{-7} , of 1 killed bee.

the extracts at high speed, resuspending the pellets in 1 ml of PB and examining the suspensions for a virus peak in the analytical centrifuge by Schlieren optics. One bee killed by Arkansas bee virus contained about 10^9 infective doses (Fig. 4*f, i*). Furthermore, virus was extracted from the live bees in increasing amounts as injected inocula were diluted, till very near the end-points (Fig. 4, Expt. 1 and 2). In one test (Fig. 4, Expt. 3) every bee of 195 was

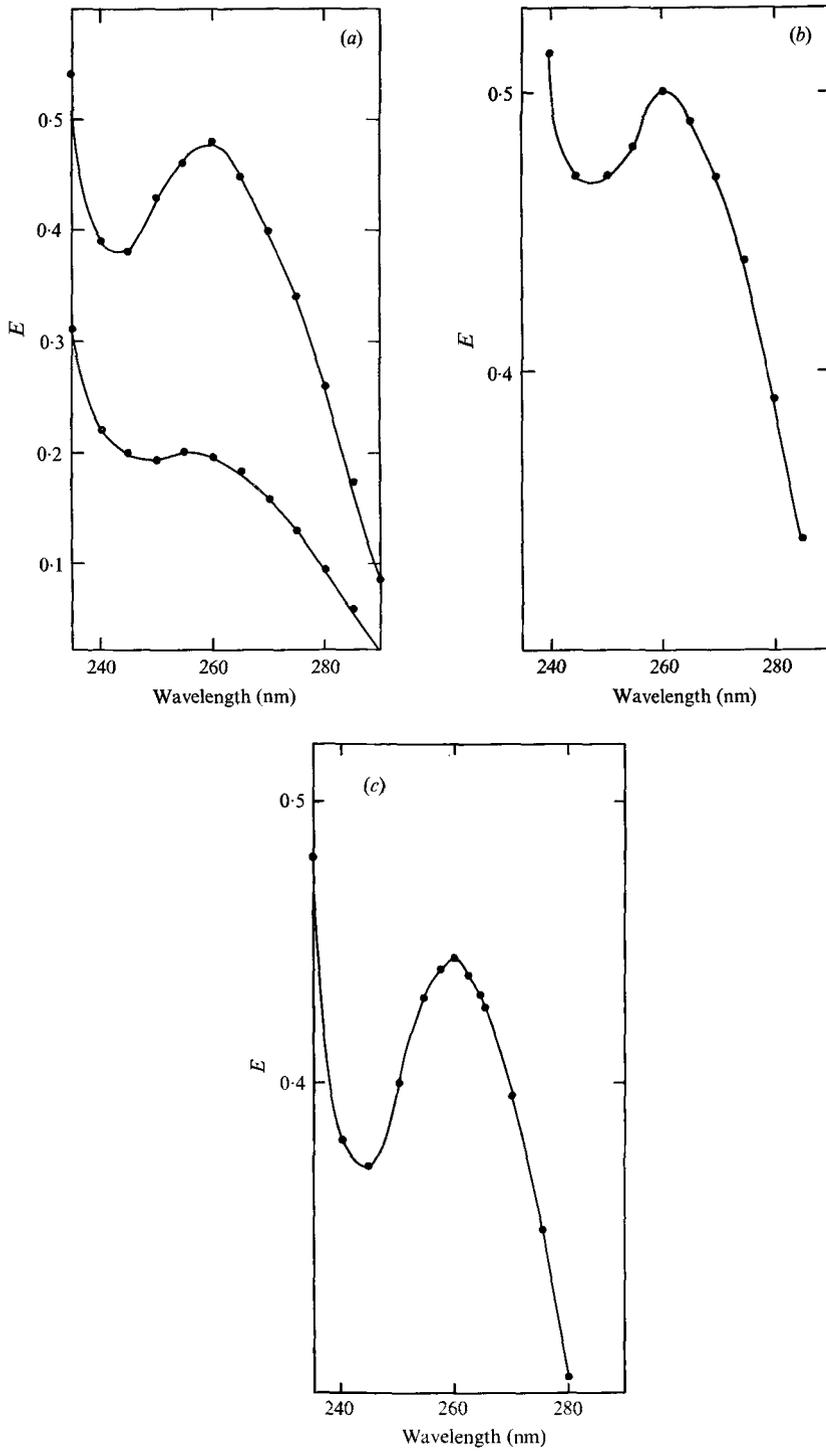


Fig. 5. Ultraviolet absorption spectra of purified virus preparations. (a) Arkansas bee virus: upper line = 128 S component; lower line = 170 S component, (b) bee virus X, (c) slow paralysis virus.

killed by injection with a moderately diluted crude extract of one bee killed by Arkansas bee virus in 1 ml, but although the virus in their heads was readily detectable serologically, there was less virus/bee than in the fewer bees (57/198) that died later when injected with the much more diluted extract. In this test the number of bees killed by the two treatments during the 18 days after injection was significantly greater ($P < 0.001$ and < 0.025 , respectively) than in the controls.

Bee virus X

When Arkansas bee virus was injected into bees in Britain and attempts made to purify it from them, a very large component appeared in sucrose gradients, in the same position as the small quickly-sedimenting component seen previously. Electron microscopy showed that this quickly sedimenting component was composed mostly of particles about 35 nm in diam. (Fig. 1*b*). They had a sedimentation coefficient of 187S, gave a single precipitin line in immunodiffusion tests against their homologous antiserum, using borate or phosphate buffer, with or without EDTA, and did not react with antisera to any other bee virus. The control bees, injected with PB, contained about the same amount of the 35 nm particles as the bees injected with Arkansas bee virus and field surveys showed that these particles occurred commonly and were frequently very numerous in live and dead bees about the end of winter. They had u.v. absorption curves typical of a nucleoprotein (Fig. 5*b*) but caused no symptoms when injected into adult bees. For this reason we refer to them as bee virus X. However, in one test, groups of bees lived only 4 days (5 replicates, s.e. (mean) 0.0) when injected with the crude extract of a field sample of bees that contained about 10^{10} particles/ml of bee virus X and about the same concentration of sacbrood virus, and to which was added 3% normal rabbit serum + 2% antisera to acute and chronic paralysis viruses. When 3% antiserum to bee virus X was added to the extract instead of normal rabbit serum, the lives of the injected bees increased to 8.2 days (5 replicates, s.e. (mean) 0.2). Other pathogenic factors may have been in the crude extract, since sacbrood virus alone takes longer than 8 days to kill adult bees (Bailey & Fernando, 1972), although electron microscopy and immunodiffusion tests detected sacbrood virus only in killed bees, most in the group taking longest to die. Therefore, bee virus X can be pathogenic and need not multiply much, at least when in combination with sacbrood virus, to have an effect on bees.

Each of many attempts failed to cause bee virus X to multiply sufficiently to be seen by electron microscopy, by injecting the virus into bees. However, when newly emerged bees were each fed between 10^6 and 10^9 particles, and then kept at 30 °C, they each contained about 10^{10} particles after 3 weeks and 10^{11} after 5 weeks, although they still appeared as healthy as control bees, which contained no particles. Very few particles were seen in extracts of similarly infected bees kept at 35 °C. Most particles, in bees from the field or in individuals that had been fed bee virus X in the laboratory, were in the abdomen and were concentrated in the gut. Sufficient virus was in the abdomen of many field bees in late winter for crude extracts to give dense precipitin lines in immunodiffusion tests.

Slow paralysis virus

During field surveys for bee virus X, we identified varying numbers of particles of other known bee viruses in the extracts. Acute bee-paralysis and chronic bee-paralysis viruses were especially common and sacbrood virus less so. About this time, Triton X-100 (see Methods) was first used in the virus purification procedure, which disintegrated particles of chronic paralysis virus. The other viruses were unaffected and were recognized by electron micro-

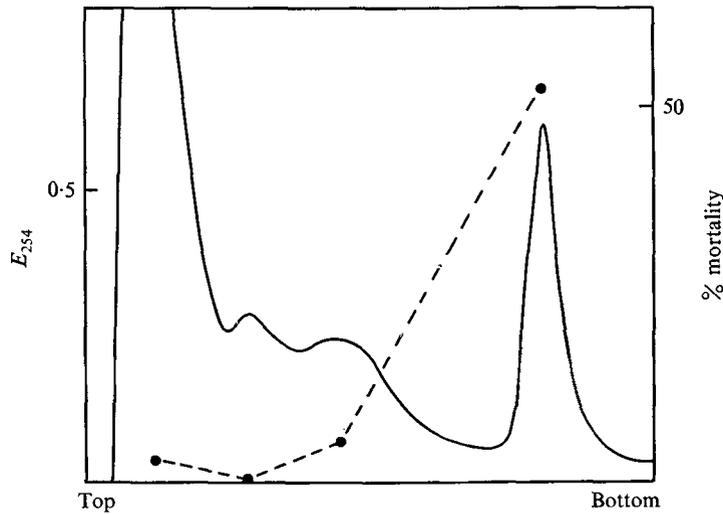


Fig. 6. Sucrose gradient analysis of bee extract containing slow paralysis virus showing distribution of E_{254} , and infectivity (● --- ●) of 1 ml fractions.

scopy, serology and sedimentation coefficients. Occasionally, however, we noticed isometric particles, 30 nm in diam., that did not react with any of the antisera tested and that had sedimentation coefficients, in resuspended pellets from clarified crude extracts, between 146S and 163S, increasing as preparations were purified by extra cycles of sedimentation and clarification. When injected with preparations of these particles and antisera to neutralize all the other known bee viruses, adult bees died after about 12 days at 30 or 35 °C, typically suffering a paralysis of the anterior two pairs of legs for a day or two before death. The paralysed bees each contained about 10^{12} isometric particles which were 30 nm in diam. (Fig. 1c), had u.v. absorption spectra typical of nucleoproteins (Fig. 5c) and, in sucrose gradients, gave a single component with which all the infectivity was associated (Fig. 6). We call the virus slow bee-paralysis virus to differentiate it from the comparatively quick-acting chronic and acute bee-paralysis viruses (Bailey *et al.* 1962).

Particles of slow paralysis virus cultivated in bees in the laboratory did not react with antisera to any of the other bee viruses and gave a single precipitin line in immunodiffusion tests against the homologous antiserum. However, after partial purification by differential sedimentation, or more complete purification in sucrose gradients, the particles aggregated in agar containing 0.85% sodium chloride or potassium chloride, at pH 5.8 to 8.8, sometimes producing 'Liesegang' rings (Fig. 7). Similar aggregation occurred without antiserum and the precipitate remained unchanged when the agar was repeatedly washed in distilled water. The virus particles aggregated less readily in agar containing phosphate-buffered 0.01% KCl than in agar containing borate-buffered saline, but they aggregated in agar containing either buffer + 2% EDTA. However, when mixed with an equal vol. of normal rabbit serum the particles did not aggregate in any of these agars, and then reacted clearly and specifically with homologous antiserum. The virus in crude extracts of the heads of bees that it had killed was also insensitive to salt and gave single dense precipitin lines in immunodiffusion tests.

After purification, slow paralysis virus sedimented as two components in the analytical centrifuge, one major and the other slow and very minor, possibly of empty particles, with

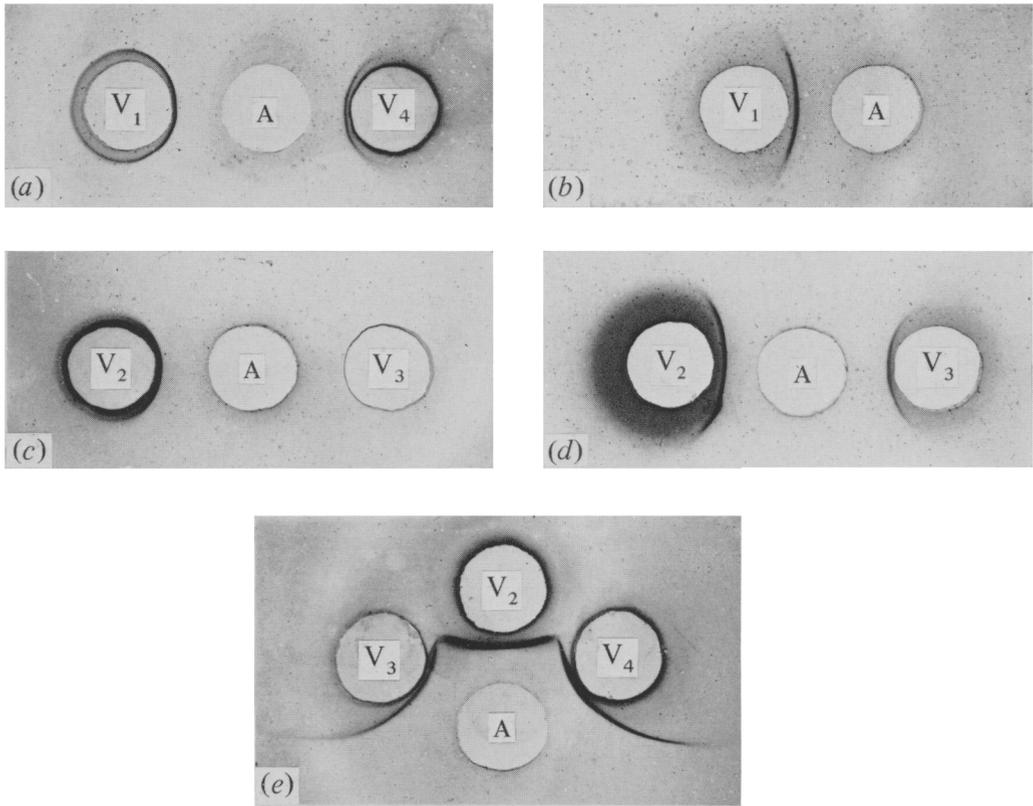


Fig. 7. Immunodiffusion tests, against homologous antiserum (A), with slow paralysis virus preparations (V_1 to V_4) mixed with equal vol. of (a, c) saline, (b, d) normal rabbit serum and (e) phosphate buffer, in agar containing (a, b) borate-buffered saline, (c, d) phosphate-buffered 0.75% KCl or (e) phosphate buffered 0.01% KCl.

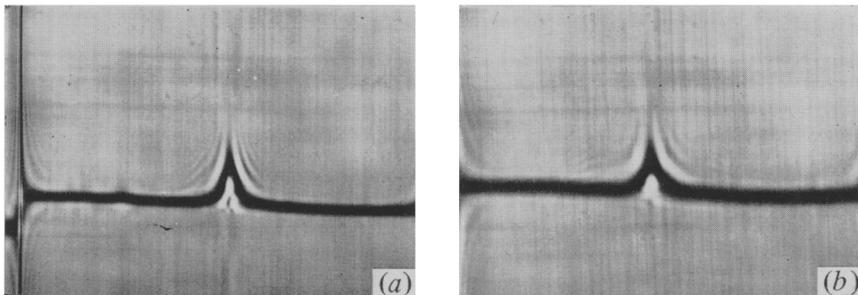


Fig. 8. Schlieren diagrams of purified slow paralysis virus, photographed after 12 min at 29000 rev/min; bar angle 45° (a) in PB 20.6 °C, (b) in PB + 0.1 M-KCl, 21 °C.

Table 1. *Characteristics of the known viruses with isometric particles of honey bees*

Virus	Size (nm)	Sedimentation coefficient	Buoyant density in CsCl (g/ml)	E_{260}/E_{280}
Acute paralysis	30	160	1.34*	1.64*
Sacbrood	30	160 (157*)	1.33*	1.67*
Arkansas				
(1) Major component	30	128	1.37	1.59
(2) Minor component	30	170	—	1.54
X	35	187	1.36	1.28
Slow paralysis	30	173-178	1.35	1.50

* Newman *et al.* (1973).

sedimentation coefficients of 173S and 84S, respectively. The same virus preparation sedimented at 178S in 0.1 M-KCl (Fig. 8) but with no indication of aggregation, as occurred in immunodiffusion tests when the agar contained the same salt solution.

All three of these newly described viruses contain RNA as indicated by positive orcinol tests and negative diphenylamine reactions (H. A. Scott and J. F. E. Newman, unpublished observations).

Table 1 gives some of the properties of all the known viruses with isometric particles.

DISCUSSION

The minor, faster-sedimenting component, observed when Arkansas bee virus was centrifuged in sucrose gradients, was probably composed of dimers. Its sedimentation coefficient of 170S is within the theoretical limit for dimers calculated from the sedimentation coefficient of the main peak (Markham, 1962). The minor component increased relative to the major one after repeated sedimentation, presumably due to concentration, and it decreased when the virus was kept a few days in solution, presumably due to disaggregation. Disaggregation also presumably occurred when preparations were made in PTA. The reason why Arkansas bee virus does not react serologically in the presence of EDTA is unknown, but the precipitation by EDTA of slow paralysis virus in agar resembled the effect of saline. This reaction of slow paralysis virus in agar containing dilute salts, its failure to disaggregate in water and its protection, presumably by the proteins, of serum or crude bee extract, somewhat resembles that of a lyophobic sol and suggests that the virus particles lose affinity for water during purification. This could explain their greatly increased sedimentation coefficient when purified.

The seemingly paradoxical increase of Arkansas bee virus in bees injected with decreasing amounts is analogous to the accumulation of more acute bee-paralysis virus in bees that survived injection with small amounts of this virus than in bees killed by injection with large amounts (Bailey & Milne, 1969). Presumably several bee viruses can multiply in some bee tissues without killing the host, while other tissues essential to the life of the individual are protected. Large inocula of Arkansas bee virus seem to overwhelm this defence, but sublethal doses are prevented or at least delayed from making fatal attacks and hindered, in proportion to the amount of virus injected, from multiplying in regions not immediately essential to the life of the individual. Therefore, Arkansas bee virus may activate defence mechanisms in proportion to the amount of virus injected.

Bee virus X multiplies slowly, and may shorten the lives of bees only slightly, except perhaps when in association with other pathogens. Nevertheless it may become important when longevity of adult bees is crucial for the survival of bee colonies, as in late winter. The natural occurrence and significance of Arkansas bee virus and of slow paralysis virus are unknown although they too probably shorten the lives of bees and may cause some of the many unexplained losses of bees.

The fact that at least six viruses of the small non-occluded type, all well differentiated from each other and unrelated serologically, occur in one insect species suggests that very many of the same type remain to be identified among insects. Possibly the gregariousness of bees enables their viruses to survive especially well, particularly as most if not all viruses can persist as inapparent infections. However, it also seems probable that many such viruses have been recognized in the bee because it has been scrutinized more than other insect species. Usually only solitary examples of similar viruses have been recognized in a very few other insect species (Newman *et al.* 1973), but five have been reported from the pine emperor moth, *Nudaurelia cytherea capensis* (Juckes, 1970), and three picornaviruses have been described from *Drosophila* spp. (Jousset *et al.* 1972).

We thank Dr H. A. Scott, University of Arkansas, and Dr D. A. Govier, Rothamsted Experimental Station, for preparing antisera and Dr Scott and Mr J. F. E. Newman, Animal Virus Research Institute, Pirbright, for determining nucleic acid types.

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(Received 25 April 1974)