STUDIES OF VIRUSES OF AUSTRALIAN HONEY BEES

by

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STATEMENT

The work reported in this thesis was done by me.

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SUMMARY

Twelve *Trigona* colonies in eastern New South Wales (NSW) and south-east Queensland and one colony in the Northern Territory were surveyed for viruses during February 1981. Virus infections were found to be less common than they are in honey bee colonies, as virus-like particles were only seen in one extract and no evidence was obtained for the presence of viruses of honey bees, nor did sacbrood virus (SBV) grow in inoculated *Trigona* pupae. These results suggested that *Trigona* is not a source of viruses for honey bees in eastern Australia.

The occurrence and distribution of viruses in colonies of the honey bee, *Apis mellifera*, was surveyed during October 1981 in north-eastern NSW and south-east Queensland, and during October 1982 in south, central and far north coastal regions of Queensland. SBV was present in 85% and 93% of fifty-two and thirty colonies inspected in the 1981 and 1982 surveys respectively. Black queen cell virus (BQCV), chronic bee paralysis virus (CBPV) and cloudy wing virus (CWV) were the only other viruses found, but they occurred less frequently than SBV.

Inapparent BQCV, cricket paralysis virus (CrPV), Kashmir bee virus (KBV) and SBV infections were found to be present in honey bee pupae from an apparently normal healthy colony throughout the year, but inapparent BQCV, KBV and SBV infections were more common in spring and

summer. The infections were activated by injecting individual pupae with 10 mM potassium phosphate buffer (PP buffer), then incubating them at 35°C for 3 days. Inapparent infections of SBV, but not the other viruses, were sometimes activated merely by incubating pupae at 35°C. When activated, only one virus was ever detected in an individual pupa.

The activation of inapparent BQCV, KBV and SBV infections in honey bee pupae was suppressed by injecting pupae with dilute specific antisera. However, greater proportions of inapparent SBV infections were activated in groups of pupae in which inapparent KBV infections were suppressed than were activated in pupae in which these infections were not suppressed. In addition, greater proportions of inapparent BQCV infections were activated in groups of pupae in which inapparent KBV and SBV infections were suppressed than were activated in pupae in which these virus infections were not suppressed. These results suggest an individual pupa may be inapparently infected with more than one virus but that when KBV is activated, it suppresses SBV and BQCV replication, and that when KBV is absent or suppressed, SBV may be activated and will suppress BQCV, and that BQCV will only replicate when KBV and SBV are absent or suppressed.

Inapparent KBV and SBV infections were established in young larvae when extracts containing particles of each virus were added to their food. There was no significant difference in the susceptibility of 1, 2, 3 and 4-day-old

larvae for either virus, but these were significantly more susceptible for SBV than were 5-day-old larvae. Susceptible larvae that became inapparently infected developed normally into inapparently infected pupae, and later, emerged as inapparently infected worker bees. Inapparent infections in newly emerged bees were activated by injecting the bees with PP buffer.

Despite extensive studies using SBV isolates from widespread localities and a number of discriminatory techniques, no consistent strain differences correlated with geography were found. The use of techniques to compare KBV coat proteins to distinguish strain differences gave equivocal results, as the proteins underwent rapid degradation, however, preliminary results suggest that less equivocal results may be obtained when using restriction endonucleases to obtain spectra of fragments of complementary DNA prepared against the genomes of KBV isolates.

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CHAPTER 1

INTRODUCTION

1.1 HONEY BEE VIRUSES

Many viruses of insects have particles that become embedded in crystalline inclusion bodies, or 'polyhedra', in the late stages of the replication cycle. The particles survive in these polyhedra until they are eaten by, and infect the gut cells of, susceptible hosts. These viruses are generally called 'occluded viruses' and often have a limited host-range. The major crystalline protein of these polyhedra is encoded by the infecting virus and the polyhedra are usually large enough to be seen in a light microscope (Harrap and Payne, 1979; Mathews, 1982). Other insect viruses have particles which do not become embedded in polyhedra. Many of these 'non-occluded' viruses have been isolated from insects and partly characterized. Most have small isometric particles that contain a single strand of RNA as their genetic material (Mathews, 1982).

The viruses that have been isolated from the honey bee, *Apis mellifera* L., are mainly non-occluded. The majority have been classified in the 'small RNA virus' group (Harrap and Payne, 1979; Mathews, 1982), the exception is bee virus F (BVF), which has poxvirus-like characteristics (Bailey,*et al.*,1981b). Most of the small RNA

viruses of honey bees are similar to the vertebrate picornaviruses and some physico-chemical properties of their particles are shown in Table 1.1. Properties of the particles of the honey bee viruses which are not picorna-like viruses, are listed in Table 1.2.

All the known viruses of honey bees, except BVF, were first isolated by workers at Rothamsted Experimental Station in England. At least nine have since been detected in Australian honey bees. These are: acute bee paralysis virus (ABPV) (Reinganum, 1968); black queen cell virus (BQCV) (Bailey, 1981; Hornitzky, 1982, 1984); bee virus X (BVX) (Anderson, 1980); bee virus Y (BVY) (Bailey, 1981); BVF (Bailey, 1981); chronic bee paralysis virus (CBPV) (Hornitzky, 1982, 1984; Reinganum, 1968); cloudy wing virus (CWV) (Bailey, 1981; Hornitzky, 1982, 1984); slow bee paralysis virus (SBPV) (Bailey, 1982); and sacbrood virus (SBV) (Hornitzky, 1981, 1982, 1984). In addition, Kashmir bee virus (KBV), first isolated from the eastern hive bee, *A. cerana* F., by Bailey and Woods (1977b) has been detected in honey bees from Australia (Bailey *et al.*, 1979; Rhodes, 1984; Rhodes and Teakle, 1978).

SBV and CBPV have been found in honey bees from several countries (Bailey, 1981). The diseases they cause occasionally result in severe economic losses to apiarists. Hence these viruses and aspects of their diseases have been studied in some detail, mainly in Europe and the United Kingdom. However, most of the other viruses of honey bees cause no obvious harm to colonies, are not regarded as

TABLE 1.1 Some physico-chemical properties of the particles of honey bee picorna-like viruses.

PARTICLE PROPERTIES	VIRUS* KBV	SBV	ABPV	BQCV	EBV
Size (nm)	30	30	30	30	30
$S_{20}W$	171-178	160	160	151	165
CsCl Density (g/cm^3)	1.37	1.35	1.37	1.34	1.37
No. of structural proteins	3	3	2	3	3
M.W. of structural proteins (Kd)	24.5, 37.3, 41.1.	25.0, 28.0, 31.5.	23.5, 31.5.	29.0, 32.0, 34.0.	24.9, 30.0, 41.0.
A_{260}/A_{280}	1.60	1.82	1.79	1.71	1.74
RNA strandedness	ss	ss	ss	ss	ss
Base Ratio (A:C:G:U)	ND+	32.1:17.9: 19.1:30.9.	30.3:20.5: 18.8:30.4.	ND	ND

*: KBV = Kashmir bee virus; SBV = sacbrood virus; ABPV = acute bee paralysis virus; BQCV = black queen cell virus; EBV = Egypt bee virus.

+: ND = Not determined.

References:

Bailey and Woods (1974);
Bailey and Woods (1977a);
Bailey and Woods (1977b);
Bailey *et al.*, (1963);
Bailey *et al.*, (1964);
Bailey *et al.*, (1976);
Bailey *et al.*, (1979).

TABLE 1.2 Some physico-chemical properties of the particles of non-picorna-like honey bee viruses.

PARTICLE PROPERTIES	VIRUS*							
	SBPV	ABPV	CBPV	CBPVAV	CWV	BVX	BVY	BVF
Size (nm)	30	30	20x30-60	17	17	35	35	150x450
Symmetry	isometric	isometric	anisometric	isometric	isometric	isometric	isometric	brick-shaped
$S_{20}W$	172-178	128-170	82, 97-106, 110-124, 125-136.	41	49	187	187	ND+
CsCl density (g/cm^3)	1.37	1.37	1.33	1.385	1.383	1.355	1.347	1.28
No. of structural proteins	3	1	1	1	1	1	1	~12
M.W. of structural proteins (Kd)	27.0, 29.0, 46.0.	41.0	23.5	15.0	19.0	52.0	50.0	13.0-17.0
A_{260}/A_{280}	1.50	1.50, 1.59	ND	1.24	1.34	1.28	1.24	ND
Nucleic Acid (strandedness)	ssRNA	ssRNA	ssRNA	ssRNA	ssRNA	ssRNA	ssRNA	dsDNA
Base Ratio (A:C:G:U)	ND	ND	24:28: 20:28	ND	ND	ND	ND	ND

*: SBPV = slow bee paralysis virus; ABPV = acute bee paralysis virus; CBPV = chronic bee paralysis virus; CBPVAV = chronic bee paralysis virus associate virus; CWV = cloudy wing virus; BVX = bee virus X; BVY = bee virus Y; BVF = bee virus F.

+: ND = Not determined.

References: Bailey and Woods (1977a); Bailey *et al.*, (1968); Bailey *et al.*, (1976); Bailey *et al.*, (1980a); Bailey *et al.*, (1980b); Bailey *et al.*, (1981b); Ball *et al.*, (1985); Clarke (1977, 1978).

economically important, and consequently have not been well studied.

When I commenced the work reported in this thesis, little research had been done on viruses of Australian honey bees. Therefore, I began by surveying bee colonies in eastern Australia to determine the occurrence and distribution of viruses. The surveys which I report in this thesis, and those done by Hornitzky (1982, 1984, 1985), indicate that SBV and KBV are the most common and widely distributed viruses in Australian honey bees. Therefore, as background, I will briefly review the research that has been done on these viruses by workers in other parts of the world.

1.2 SACBROOD VIRUS

Sacbrood disease of honey bees was first reported in 1857 by Langstroth (Gochnauer, 1978), but White (1917) was the first to describe the symptoms and to show that it was caused by a pathogen that could pass through a 'bacteria-proof' filter. White noted that healthy larvae pupated shortly after being sealed in their cells, whereas larvae with sacbrood failed to pupate and remained stretched on their backs with their heads pointing towards the cell capping. The unshed final larval cuticle became a transparent sac and fluid accumulated between this and the epidermis of diseased larvae. Then, beginning at the head and spreading posteriorly, their body colour gradually

changed from a pearly white to pale yellow. Shortly afterwards, the larvae died. If diseased larvae were not removed from the colony by nurse bees, they eventually dried to a dark brown, 'gondola-shaped' scale which was not infectious. White (1917) also reported that larvae first showed sacbrood symptoms about 6 days after becoming infected.

Despite the report by White (1917), apiarists came to believe that sacbrood was a non-infectious, hereditary fault known as 'addled brood'. This belief resulted from studies showing that the disease did not spread when combs containing diseased larvae were placed into healthy colonies (Tarr, 1937). However, the confusion about the disease was partly clarified when electron microscopy was applied to the study of bee diseases. Steinhaus (1949) found isometric particles about 60 nm in diameter in extracts of larvae with sacbrood but this finding was disputed when Brcak *et al.*, (1963) reported finding 30 nm diameter particles in larvae with sacbrood. The confusion was finally resolved when Bailey *et al.*, (1964) applied 'Koch's Postulates' and showed that sacbrood was caused by a virus with particles that were isometric and 30 nm in diameter. Hitchcock (1966) showed that healthy bee colonies did not develop symptoms of sacbrood when comb with diseased larvae was transferred to them. The isolation of SBV led to detailed studies of its particles, and some of their physico-chemical properties are listed in Table 1.1.

SBV replicates in the cytoplasm of cells of the fat, muscle, trachea and dermal glands of larvae, and in the mandibular and hypopharyngeal glands, abdominal ganglia and brains of worker bees (Bailey, 1968; Bailey and Fernando, 1972; Lee and Furgala, 1967a, 1967b; Mussen and Furgala, 1977). The ecdysial fluid in the sac surrounding the pupal integument of a diseased larva contains many SBV particles (Fernando, 1972). It has been suggested that infected larvae are unable to shed their last cuticle because the virus damages dermal glands which produce chitinase, the enzyme which normally breaks down the cuticle and allows pupation to occur (Bailey, 1976; Fernando, 1971, 1972; Mussen and Furgala, 1977).

Two-day-old worker larvae are most susceptible to sacbrood (Bailey, 1981). They become infected after eating contaminated honey or 'royal jelly', which is a proteinaceous secretion produced by the hypopharyngeal and mandibular glands of young nurse bees (Laidlaw, 1979). These nurse bees become infected after eating pollen collected and contaminated by infected foraging bees (Bailey and Fernando, 1972). It is not known how long SBV particles remain infective in pollen.

A single larva infected with SBV contains enough particles to infect all the larvae of more than 1000 colonies (Bailey, 1969), but in most colonies, sacbrood generally remains uncommon. This probably is due, in part, to two inherited, behavioural traits which enable worker bees to limit the spread of the disease. One of these

traits is that larvae, in the early stages of infection, are recognized and removed from the colony by nurse bees before they have produced large concentrations of SBV particles in their bodies. The other is that nurse bees do not remove dead larvae until at least 3 weeks after they have died, when the SBV particles in them have become non-infectious (Bailey, 1971; White, 1917). When either of these strategies fail, nurse bees become infected when cleaning the hive (Bailey, 1981). Even then, the spread of virus appears to be restricted by subtle, virus-induced changes in the behaviour of infected workers (Bailey and Fernando, 1972; Fernando, 1972). These bees cease to eat pollen, to attend the queen or drones, or to rear brood. They are more susceptible to chilling and try to fly and forage sooner. However, they show altered foraging behaviour by collecting mainly nectar, not pollen. The few infected bees that do collect pollen add secretions from the mandibular and hypopharyngeal glands to their pollen loads in the normal manner and thereby contaminate the pollen with SBV particles. Bailey and Fernando (1972) concluded that the altered behaviour probably limits the spread of the disease because nectar collected by infected bees usually contains less than 10^5 particles/ml, too few to infect larvae or nurse bees; the median lethal dose for highly susceptible 2-day-old larvae is 10^5-10^6 particles (Bailey *et al.*, 1964), and for nurse bees 10^8-10^9 particles (Bailey, 1969). By contrast, the pollen occasionally collected by infected foraging bees contains about 10^6

particles (Bailey, 1967), and thus may be infectious to very susceptible nurse bees.

SBV is thought to persist from year to year in adult worker bees, in which it multiplies without causing symptoms (Bailey, 1968, 1969). Outbreaks of sacbrood are common during the spring, when colony populations are increasing, and also during prolonged drought (Bailey, 1981). During these periods there is often a shortage of young workers to attend developing larvae. Therefore, older worker bees which are possibly infected resume rearing duties, and this increases the likelihood of contact between developing larvae and infected adults. During late spring and summer, when the division of labour in a colony is well established, the incidence of sacbrood decreases.

It is not known how SBV is transmitted between colonies. Shimanuki (1978) however has suggested that SBV may be transmitted from colony to colony by robber bees. Bees from an infected colony that visit neighbouring colonies, commonly called 'drifting bees', might also transmit SBV to those colonies.

1.3 KASHMIR BEE VIRUS

The particles of KBV are similar to those of SBV in that they are isometric and about 30 nm in diameter. The virus was first isolated by Bailey and Woods (1977b) from *A. mellifera* pupae that died after being inoculated with

extracts from *A. cerana* from Kashmir. It has also been isolated directly from diseased adults of *A. cerana* from Kashmir (Bailey, 1981), and from Mahableshwar, India (Bailey *et al.*, 1979).

Bailey *et al.*, (1979) detected three strains of KBV in honey bees from Australia. They were distinguished from one another and from an isolate from Kashmir by serology and by the size and stability of their coat proteins. These Australian strains were reported to be associated with severe mortality of adult bees in the field (Bailey, 1981) and one strain was associated with mortality of larvae (Rhodes and Teakle, 1978).

Since its initial isolation, KBV has been frequently detected in Australia (Dall, 1985; Hornitzky, 1981, 1982, 1984, 1985). The virus is not known to occur naturally in honey bees from the northern hemisphere, and Bailey *et al.*, (1979) suggested that, as the Australian strains showed serological differences, KBV may be endemic in south-east Asia and Australia, possibly as a pathogen of another insect species, perhaps an insect related to honey bees, such as the Australian native bee *Trigona*, from which it only occasionally spreads to honey bees. *Trigona* bees are small, dark colonial insects, commonly called 'sweat flies', and are found mainly in northern Australia. They live in large colonies and have a differentiated caste system. Some Australian species are also common to the Indo-Malayan region (Mitchener, 1965). In 1980 I was unable to isolate viruses of honey bees from eleven *Trigona*

colonies (Anderson, 1980), but the sites sampled during that study may not have been representative, as they were restricted to *Trigona* colonies in the Brisbane area of Queensland. In Chapter 3 of this thesis, I report further attempts to detect viruses in *Trigona*. Figures 1.1 and 1.2 show the queen and adult worker of *T. carbonaria* Smith, and those of *A. mellifera* for comparison.

Rhodes and Teakle (1978) reported that 'KBV disease' killed honey bee larvae in colonies in south-east Queensland in 1977-1978, and described the symptoms shown by the diseased larvae from which KBV particles were isolated. These ranged from symptoms resembling sacbrood disease to those described by White (1917) for European foulbrood (EFB) disease, which is caused by the bacterium *Melissococcus pluton* (Bailey and Collins, 1982). However, Rhodes and Teakle (1978) reported that larvae with KBV disease differed from larvae infected with sacbrood or with EFB in that they retained their shape and were pale yellow immediately after dying. Later their skin toughened, their body contents liquified, and they dried into the base of their cells, their tough skin becoming brown or black. Diseased colonies eventually recovered. Some doubt exists as to whether the symptoms reported by Rhodes and Teakle (1978) were actually caused by KBV as their attempts to complete 'Koch's Postulates' were unsuccessful.

A recent paper by Dall (1985) described some interesting studies on the ecology of KBV in Australian

honey bees. These will be discussed and compared with the results of my studies of KBV in Chapter 4.

Some of the biochemical properties of KBV particles (Kashmir strain) are listed in Table 1.1.

1.4 STUDIES REPORTED IN THIS THESIS

I began my studies by surveying honey bee colonies in eastern Australia to determine the distribution and incidence of viruses. The results of these surveys are given in Chapter 3. They indicated that viruses were widely distributed in honey bees but that they were not confined to individuals showing symptoms. Therefore I made a major study of the factors affecting inapparent infections, particularly of pupae, and this study is reported in Chapter 4. The surveys also showed that sacbrood, which had been recorded but considered of little importance in New South Wales before 1976, was widely distributed. This change may reflect the emergence, or importation and spread, of a more virulent SBV strain than that previously present. Thus, I looked for differences between various isolates of SBV from around Australia, and report these studies in Chapter 5. Also reported in Chapter 5 is a study of differences between isolates of KBV.

While studying inapparent infections and strain variation, I also investigated:

1. The potential value of new, sensitive serological techniques for detecting honey bee viruses. The results of this work have been published (Anderson, 1984, Appendix A);

2. Whether honey bee viruses could replicate in cultured cells. With the help of Drs A. Braithwaite, L. Dalgarno, T.D.C. Grace, and R.J. Mahon, I could not detect any replication of KBV or SBV in Hep2 human carcinoma (Flow Laboratories), B54 mouse fibroblast (C57 black-6J x AKR-J strain) or in *Aedes albopictus* or *Antheraea eucalyptii* cultured cells, nor could I confirm the claim by Smirnova (1966) that SBV replicated in chicken embryo fibroblasts. Therefore I did not pursue this line of research.

Most of the methods and materials used in my studies are reported in the following chapter. However, some specific methods are described in the relevant sections of other chapters.

Figure 1.1 A queen and adult worker of the Australian native bee, *Trigona carbonaria*.

Figure 1.2 A queen and adult worker of the honey bee, *Apis Mellifera*.

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF SPECIMENS

Dead bees collected from *Trigona* and honey bee colonies were put in small vials and transported, on ice, to the laboratory where they were stored at -20°C.

2.2 BIOASSAY

Virus preparations were propagated in adult worker bees and bee pupae less than two days old. The age of honey bee pupae is most easily assessed from the colour of their eyes; up to 2 days of age they are white, on day 3 they are pink, and thereafter they darken, until finally they become a brownish-black colour (Waller, 1980). White-eyed pupae were located among sealed brood by removing their wax cell cappings, and each pupa was carefully withdrawn from its cell using bent forceps to grip behind the head vertices. White-eyed *Trigona* pupae were also used to attempt to propagate honey bee viruses.

Virus extracts were injected into the haemocoel of individual bees through a dorso-lateral intersegmental

membrane of the abdomen. *Trigona* pupae were injected using a sterile glass capillary connected to a timer-pulsed nitrogen supply and with the aid of a dissecting microscope. Honey bee pupae were injected using a sterile unit consisting of a micrometer syringe connected to a 'bacteria-proof' filter (0.22 μm, Millex) and a 30 gauge needle. Inoculated pupae were incubated at 35°C in a container with filter paper, wetted with 12% glycerol to humidify the air, until they were moribund or dead. Mature adult worker honey bees were injected in the same way as pupae, except they were anaesthetised with carbon dioxide prior to injection, then kept at 35°C in small cages (fifteen individuals per cage) supplied with a 60% sucrose solution and water in gravity feeders.

2.3 PREPARATION OF VIRUS EXTRACTS

Virus extracts were prepared using a similar method to that described by Bailey *et al.*, (1980a). Groups of 30 honey bees or 150 *Trigona* bees were ground in a mixture of 27 ml of 10 mM potassium phosphate buffer, pH 6.7 (10 mM KH_2PO_4, 4 N KOH, hereafter called PP buffer), and 3 ml of 20 mM sodium diethyldithiocarbamate (DIECA), shaken with 3 ml of ether, then with 3 ml of carbon tetrachloride (CCl_4), and centrifuged at 3000g for 10 min. The supernatant was centrifuged at 75,000g for 3 hrs., the pellet resuspended in PP buffer, and centrifuged at low speed to remove debris. These preparations (hereafter called 'group

preparations') were usually further purified using 10-40% sucrose gradients prepared in PP buffer by layering 10%, 20%, 30% and 40% sucrose solutions in cellulose nitrate tubes. These tubes were kept at 4°C overnight for the gradients to form. One ml of virus suspension was layered on top of the gradient and centrifuged at 180,000g for 45 min. The virus particles, which were detected as a broad, light scattering band, were removed into PP buffer, and pelleted by centrifuging again at 180,000g for 2 hrs. They were resuspended in 0.2 ml of PP buffer.

Extracts were also prepared from individual honey bees (hereafter called 'individual preparations'). These were obtained in a similar way to group preparations, except that each individual was ground in 0.9 ml of PP buffer and 0.1 ml 20 mM DIECA, shaken with 0.1 ml CCl_4, and centrifuged at 10,000g for 30 seconds to clear debris. The supernatant extract was tested for the presence of viruses and the pellet was discarded.

2.4 SOURCES OF ANTISERA

An antiserum prepared by immunizing a rabbit with the particles of cricket paralysis virus (CrPV) (antiserum reciprocal titre 32) was kindly provided by Dr A.J. Gibbs. This serum had a titre of 1:4 against an antigen from apparently healthy honey bee pupae. However, when diluted to 1:32, the serum reacted only with CrPV particles.

Antisera were also kindly provided by Dr L. Bailey, Rothamsted Experimental Station, England. The sera had been prepared by immunizing rabbits with the particles of acute bee paralysis virus (ABPV; antiserum reciprocal titre 1024), black queen cell virus (BQCV; 512), bee virus X (BVX; 64), bee virus Y (BVY; 64) chronic bee paralysis virus (CBPV; 64), cloudy wing virus (CWV; 128), slow bee paralysis virus (SBPV; 256), Kashmir bee virus (KBV; 64), and sacbrood virus (SBV; 32). At the antiserum dilution end-point, each serum reacted only with the virus particles against which it was prepared and no cross reactions were ever detected, except that the CBPV antiserum had a titre of 1:32 against antigens from apparently healthy bees.

Antisera against BQCV, CBPV, KBV and SBV particles were each produced in rabbits by immunizing them each with three 1.0 ml injections of purified particles (approx. 1.0 mg/ml; determined by light absorbtion at 280 nm) over a period of 5 weeks. The first injection was intravenous and the others intramuscular, using virus particles emulsified in Freund's complete adjuvant. Blood samples were removed from the ear vein of each rabbit 1-2 weeks after the final injection, and serum was separated from clotted blood. Sodium azide (0.1 mg/ml) was added to each serum as preservative. All undiluted sera, and sera diluted with an equal volume of 0.9% saline, reacted weakly with preparations of healthy honey bees in conventional Ouchterlony gel diffusion tests (described below). At dilutions greater than this, each reacted only with

particles of the homologous, not heterologous, virus. The reciprocals of the homologous titres were the same as those obtained for Dr Bailey's antisera.

Each virus preparation used to immunize a rabbit was obtained from thirty individual preparations which were individually checked, pooled and purified, as described for group preparations, then further purified using sucrose gradients. The BQCV and SBV preparations were purified from diseased larvae collected from honey bee colonies in Richmond, New South Wales (NSW). KBV was purified from inapparently infected pupae obtained from a colony in Canberra, in The Australian Capital Territory; the pupae became moribund when injected at the white-eyed stage with 10 µl of PP buffer (see Chapter 4). CBPV was purified from adult worker bees that had died of chronic paralysis disease, and which were kindly supplied by Mr M. Hornitzky, Department of Agriculture, Veterinary Research Station, Glenfield, NSW. The identity of virus particles in purified extracts was determined by conventional gel diffusion serological tests and negative staining electron microscopy (described below), using Dr Bailey's antisera.

2.5 VIRUS DETECTION

2.5.1 Electron Microscopy

Individual and group preparations were examined in the electron microscope after staining with an equal volume of

2% ammonium molybdate and spraying onto carbon coated Formvar films supported on electron microscope grids.

2.5.2 Serological Methods

2.5.2.1 Gel Diffusion

Conventional gel diffusion tests (Mansi, 1958) were used to detect viruses in individual and group preparations. Tests were done using 4.0 ml of 0.75% agar or agarose in 50 mM potassium phosphate buffer, pH 6.7 (50 mM KH_2PO_4, 20 N KOH), containing 5 mM EDTA and 0.2% sodium azide on a 40 mm x 80 mm sheet of 'Gel-bond' (Pharmacia). The reactants were placed in circular wells 4 mm in diameter in a hexagonal pattern and 2 mm apart. After 12 hours incubation at room temperature in a moist atmosphere, the gel was inspected for the presence of precipitin lines.

Preliminary experiments showed that gel diffusion tests were able to detect particles in individual preparations of BQCV, CBPV, KBV or SBV, diluted to 10^{-1} and occasionally 10^{-2}, whereas most group preparations had dilution end-points of 10^{-2} and occassionally 10^{-3}. Assuming that a threshold of 10^{12} to 10^{13} small spherical virus particles are needed for a reaction in a gel diffusion test (Gibbs and Harrison, 1976), individual preparations probably contain about 10^{13} to 10^{14} virus particles.

2.5.2.2 Enzyme Linked Immuno Sorbent Assay (ELISA)

An indirect ELISA method (Koenig and Paul, 1982) was used to detect KBV and SBV in individual preparations. In outline, wells of a microtitre haemaglutinin plate were coated with $F(ab')_2$ IgG fragments, which trapped homologous antigen in the preparations. Bound antigen was detected by specific rabbit IgG which in turn was detected by sheep anti-rabbit IgG conjugated to urease. The presence of urease was detected by providing it with a substrate which changed colour.

The IgG for ELISA was purified by the method described by Clark and Adams (1977). A 20 ml mixture containing 1.0 ml of specific rabbit antiserum, 9 ml distilled water, and 10 ml saturated neutralised ammonium sulphate solution, was prepared and left for 1 hr at room temperature, then centrifuged at 2,000g for 30 min to collect the IgG, which was resuspended in 2 ml of half-strength phosphate buffered saline, pH 7.4 (PBS, full-strength PBS = 137 mM NaCl, 2 mM KH_2PO_4, 16 mM Na_2HPO_4, 3 mM KCl). This was dialyzed 3 times against half-strength PBS overnight. The concentration of IgG was measured spectrophotometrically using light of 280 nm wavelength and the equation: IgG (mg/ml)=$O.D._{280nm}$ x dilution of IgG/1.4. Then, the IgG was adjusted to 1 mg/ml and stored in silicone treated glass tubes at $-18^{o}C$.

Urease conjugated with sheep anti-rabbit IgG and a proprietary urease substrate solution were purchased from

The Commonwealth Serum Laboratories (CSL), Melbourne, and stored at -18°C and 4°C respectively.

To prepare $F(ab')_2$ fragments, 0.25 ml of partially purified specific IgG (4 mg/ml) in 0.75 ml of 70 mM sodium acetate and 50 mM sodium chloride was digested with 22.5 µl of pepsin suspension (1 mg/ml) by incubating overnight at 37°C (Adams and Barbara, 1982). Digestion products were removed by dialyzing against 3 changes of PBS buffer.

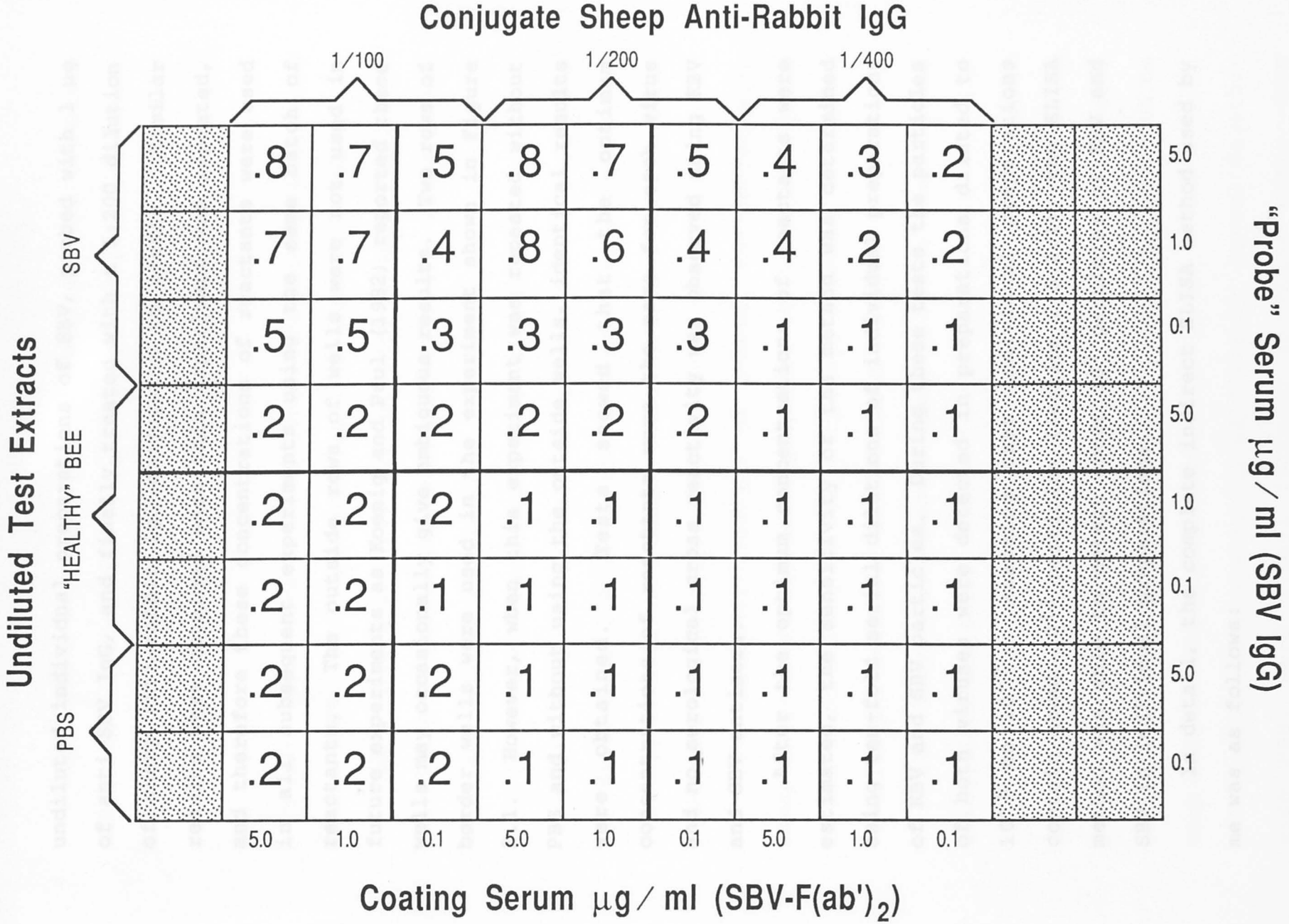
Tests were first done to determine the optimum conditions for detecting SBV and KBV in individual preparations. The general strategy of these tests was as follows. The optimum concentrations of reactants were determined in micro-titre plates using a chequerboard arrangement of combinations of PBS, undiluted individual preparations of SBV or KBV particles, or a 'healthy' bee pupa, and dilutions of coating serum (SBV-$F(ab')_2$ or KBV-$F(ab')_2$ fragments), probe serum (SBV IgG or KBV IgG), and conjugated sheep anti-rabbit IgG. The CSL urease substrate was always used undiluted. Absorbances were measured using a wavelength of 410 nm with a Titertek Multiskan MC (Flow Laboratories Ltd, North Ryde NSW, Australia), adjusted to an O.D. scale of 0 to 1.0, and the ratios of absorbances were then compared between wells. The results from a typical plate are shown in Figure 2.1. These clearly show the ratios of absorbances between wells containing healthy bee extracts or PBS, and wells containing undiluted individual SBV preparations were greatest when using wells coated with 5 µg SBV-$F(ab')_2$ fragments, then treated with

FIGURE 2.1 Optical densities* obtained from ELISA tests comparing the reaction of phosphate buffered saline (PBS), "healthy" bee extract and bee extracts containing sacbrood virus (SBV), with different concentrations of coating serum (SBV-$F(ab')_2$), "probe" serum (SBV IgG) and conjugate IgG^+.

* : Optical densities were adjusted as described in the text.

$^+$: See text for experimental details.

Note: Shaded wells were not used in this experiment.


Conjugate Sheep Anti-Rabbit IgG
1/100
1/200
1/400
Undiluted Test Extracts
SBV
"HEALTHY" BEE
PBS
.8 .7 .5 .8 .7 .5 .4 .3 .2
.7 .7 .4 .8 .6 .4 .4 .2 .2
.5 .5 .3 .3 .3 .3 .1 .1 .1
.2 .2 .2 .2 .2 .2 .1 .1 .1
.2 .2 .2 .1 .1 .1 .1 .1 .1
.2 .2 .1 .1 .1 .1 .1 .1 .1
.2 .2 .2 .1 .1 .1 .1 .1 .1
.2 .2 .2 .1 .1 .1 .1 .1 .1
5.0 1.0 0.1 5.0 1.0 0.1 5.0 1.0 0.1
5.0
1.0
0.1
5.0
1.0
0.1
5.0
0.1
Coating Serum µg / ml (SBV-F(ab')2)
"Probe" Serum µg / ml (SBV IgG)

undiluted individual preparations of SBV, probed with 1 µg of anti-SBV IgG, and finally treated with a 1:200 dilution of sheep anti-rabbit IgG conjugated to urease. Similar results were obtained when this experiment was repeated, and therefore these concentrations of reactants were used in all subsequent experiments using the same batch of reactants. The outside rows of wells were not used in future experiments as Koenig and Paul (1982) reported these wells may occasionally give ambiguous results. Two rows of border wells were used in the experiment shown in Figure 2.1. However, when this experiment was repeated without PBS and without using the outside wells, identical results were obtained. Tests showed that the optimum concentrations of reactants were the same for each virus and no serological cross reactivity was observed using KBV and SBV antisera.

After the optimum concentrations of reactants were estimated, the sensitivity of the method was determined using ten-fold serial dilutions of individual preparations of KBV and SBV particles. During these tests the particles of both viruses were detected in preparations diluted to 10^{-6} to 10^{-7}. Assuming that individual preparations contain about 10^{13} particles (Section 2.5.2.1), the ELISA method was able to detect about 10^{7} particles of KBV and SBV in individual preparations.

In detail, the complete indirect ELISA method used by me was as follows:

(1) Wells in polystryene microtitre trays (Dynatech M129B) were coated with 200 µl aliquots of purified rabbit anti-KBV or anti-SBV $F(ab')_2$ fragments (5 µg/ml) diluted in 50 mM sodium carbonate (pH 9.6) by incubating at 37°C for 2 hrs.

(2) Trays were rinsed 3 times with phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween-20 (PBS-T).

(3) Aliquots (200 µl) of test samples in PBS-T containing 2% polyvinylpyrrolidone (PVP) were added to the wells and left at 4°C overnight.

(4) Trays were rinsed 3 times with PBS-T.

(5) Aliquots (200 µl) of specific rabbit anti-virus IgG diluted to 1.0 µg/ml in PBS-T containing 2% PVP and 0.2% egg albumin were added to each well and incubated for 3 hrs at 37°C.

(6) Aliquots (200 µl) of urease, conjugated with sheep anti-rabbit IgG immunoglobulins (CSL), diluted 1:200 in PBS-T containing 2% PVP + 0.2% egg albumin, were added to wells and incubated at 37°C for 3 hrs.

(7) Trays were rinsed 3 times in PBS-T, then 3 times using distilled water.

(8) Aliquots (200 µl) of undiluted urease substrate (CSL) were added to each well and incubated for 30 min at 37°C, then 10 µl of a 1% (w/v) aqueous (unbuffered) solution of sodium ethylmercuri-thiosalicylate was added to each well to stop the reaction.

Individual preparations of 'healthy' pupae, together with PBS, were always included in ELISA tests when

determining whether individual preparations were infected with KBV or SBV. However, the absorbances obtained from wells containing extracts of an apparently healthy pupa were always very low and were rarely found to be greater than those obtained from wells containing PBS. ELISA reactions which gave absorbance readings twice as great as those of the controls were considered positive reactions. To guard against false positives, each test was replicated in another microtitre tray.

2.5.3 Electrophoresis of Virus Particles

Virus particles were separated and characterized by horizontal slab electrophoresis using an electrophoresis buffer consisting of 0.1 M Tris, and 50 mM di-sodium EDTA adjusted to pH 7.0. For each experiment, 5 µl of a particle preparation, together with 5 µl of 1% phenol red tracker dye, was loaded into wells in one end of a 0.75% agarose slab gel (prepared in electrophoresis buffer, diluted 1 in 4 in distilled water) on 50 x 75 mm or 25 x 75 mm glass slides. 250 volts was then applied. Electrophoresis was terminated when the tracker dye had migrated to the end of the gel (about 60 mm), and the position of the tracker dye was then marked. Virus particles in the gels were located by examining the gel for ultra-violet (U.V.) absorbing spots on a fluorescent background, or by staining each gel in 0.25% (w/v) Coomassie Brilliant Blue (CBB) for 2 hours, followed by

destaining in methanol : acetic acid : distilled water (4.5:1:4.5). Alternatively, virus particles were located serologically (immunoelectrophoresis) by cutting long narrow troughs between the tracks of gel through which the virus particles had migrated, filling these troughs with specific virus antiserum and examining for the presence of precipitin lines which usually developed after several hours incubation at room temperature. The distance that the particles of each isolate migrated was converted to an RØ value by expressing it as a proportion of the distance (in mm) that the phenol red tracker dye had migrated.

2.5.4 Isoelectric Focussing

The isoelectric points of each isolate were estimated by electro-focussing 5 µl of each preparation in a stabilized pH gradient, from pH 3 to pH 10, using 2% ampholytes in 'Agarose IEF' (Ampholine, LKB Productor, Sweden) at 20 mA. The virus preparations were loaded into wells cut in different locations in the gel to ensure that the electro-focussed particles had equilibrated at their isoelectric points. The pH of the gel from the cathode to the anode was estimated using a pH meter and strips cut from the edge of the gel. The position of the virus particles was visualised after staining the gel with CBB, followed by destaining.

2.6 ESTIMATING MOLECULAR WEIGHTS OF VIRUS CAPSID PROTEINS

The molecular weights of the capsid proteins were estimated by the method of Laemmli (1970), using 12 cm x 12 cm vertical slab gels, each consisting of a separating gel of 15% polyacrylamide (30:1 acrylamide, bis-acrylamide (BDH)), containing 0.4% sodium dodecylsulphate (SDS), in 0.375 M Tris-HCl buffer, pH 8.8, and a 3% polyacrylamide stacking gel (20:1 acrylamide, bis-acrylamide) containing 0.4% SDS in 0.125 M Tris-HCl buffer, pH 6.8. The running buffer was 25 mM Tris containing 0.192 M glycine and 1.0% SDS, pH 8.3.

The molecular weights of the polypeptides were estimated by comparison with a line of best fit of the $\log_{10}$ of the weights of marker standards (Pharmacia) against the distance travelled (in mm) in the gels. The standards used and their molecular weights were as follows.

MARKER PROTEIN	MOLECULAR WEIGHT (daltons)
Phosphorylase B	94,000
Albumin	67,000
Ovalbumin	43,000
Carbonic anhydrase	30,000
Trypsin inhibitor	20,100
Lactalbumin	14,400

In most experiments the trypsin inhibitor (TI) marker protein produced one band in the gels. However, in some gels an additional band was produced which had a slightly

faster mobility than the TI protein. This band was probably a degradation product of one of the marker proteins, perhaps the TI protein, and hence was not used when calculating the line of best fit.

Virus particles and marker proteins were diluted in PP buffer and mixed with an equal volume of digestion mixture (2 mls stacking gel buffer, 4 mls 10% SDS, 2 mls glycerol, 1.0 ml 2-mercaptoethanol, 1.0 ml distilled water and 5 µl bromophenol blue), and boiled for 1 min. After cooling, 5 to 10 µl of each particle isolate, together with 5 µl of marker proteins, were loaded into the wells of the stacking gel and electrophoresed at 80 volts until the bromophenol blue front had migrated to the bottom of the gel. Then, each gel was stained with CBB, destained, and the molecular weights estimated.

2.7 VIRUS GENOME ANALYSIS

2.7.1 Extraction of Viral RNA

Viral genomic RNA was obtained from virus particle isolates in group preparations after purification in sucrose gradients. The RNA was extracted from the particles of each isolate as follows. Proteinase K (1.0 ml of 1.0 mg/ml) was added to 250 µl of purified virus particles in 3.75 mls of RNA extraction buffer (10 mM Tris, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2% SDS, pH 7.6) and held at 56^{o}C for 20 mins, after which 800 µl of 1.0 M NaCl and 6.0

ml of water-saturated redistilled phenol were added. This mixture was kept at 56°C for 5 mins. Then, 6.0 mls of chloroform was added and mixed by bubbling with nitrogen gas for 5-10 mins and the phases separated by centrifuging at 5,000 rpm for 5 mins. The aqueous phase was removed and the RNA precipitated overnight at -20°C after adding one-tenth volume of 3 M NaAc, pH 5.5, and 2.5 volumes of ethanol. The precipitated RNA was pelleted by centrifuging at 7,000 rpm at 4°C for 10 mins, then resuspended in 1.0 ml of 0.3 M NaAc, pH 5.5, precipitated again with ethanol, centrifuged, dried with nitrogen gas, and finally resuspended in 200 to 500 µl of distilled water.

2.7.2 Determining the Size of Genomic RNA

The sizes of the single stranded (ss) genomic RNA from virus isolates were estimated by PAGE using a 3% acrylamide-urea gel in TBE (10.5g urea and 2.5 ml of 30% (20:1) acrylamide in 10 mM Tris, 2 mM EDTA, 0.1 M boric acid, pH 8.3) prepared using 0.9 mls ammonium persulphate and 25 µl N,N,N'N'....tetramethyl-ethylenediamine (TEMED). Before electrophoresing the RNA into a vertical polyacrylamide slab gel, the RNA concentration of each isolate was estimated in a spectrophotometer at a wavelength of 260 nm. Next, the concentration of RNA of each isolate was adjusted to 100 µg/ml in distilled water and 5 µl was mixed with 5 µl of formamide solution (containing EDTA, 0.1% xylene cyanol and 0.1% bromophenol

in formamide) and loaded onto the gel. TBE was used as the running buffer and electrophoresis was at 30-35 mA for 50 mins. The gel was then stained with 0.2% ethidium bromide and the position of the bands recorded using U.V. light.

2.7.3 Obtaining Restriction Fragment Spectra (RFS's)

The four restriction enzymes used in my studies, together with their source organism and cleavage sites, were as follows:

RESTRICTION ENDONUCLEASE	BACTERIAL SOURCE	SEQUENCE AND CLEAVAGE SITE (↓) (single strand 5' → 3')
Alu I	*Arthrobacter luteus*	A G↓C T
Hae III	*Haemophilus aegyptius*	G G↓C C
Hha I	*H. haemolyticus*	G C G↓C
Sal I	*Streptomyces albus* G	G↓T C G A C

Complementary DNA (cDNA)/viral RNA hybrid duplexes were prepared by mixing 3.0 µl of viral RNA in 14 µl distilled water and 2.0 µl of either herring sperm DNA random primers (20 mg/ml) (Boehringer Mannheim) or T_{12}-T_{18} primers (2 µg/ml of $p(dT)_{12-18}$ from P-L Biochemicals). The mixture was boiled for 1 min and cooled in iced water.

Then, 2.5 µl of 10x dSA mix (500 mM Tris, pH 8.3, 40 mM dithiothreitol (DTT), 65 mM MgAc, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), 1.0 µl of 1 mM dATP (Boehringer Mannheim), 1.0 µl of reverse transcriptase (Life Sciences, USA) and 1.5 µl of dATP (Boehringer Mannheim) were added and the mixture kept at 37°C for 2 hrs, after which 1 µl of a mixture of 1.0 mM unlabelled nucleotide triphosphates (1 mM of each) (Boehringer Mannheim) were added and the mixture held at 37°C for 30 mins.

The cDNA/viral RNA hybrids were digested overnight at 37°C using 3.0 µl cDNA/viral RNA as template, 1.0 µl of 10x Hind buffer (66 mM Tris-HCl, pH 7.4, 66 mM MgCl, 500 mM NaCl and 100 mM DTT), 5 µl of distilled water and 1 µl of either Alu I, or Hae III (kindly prepared for me by Mrs Anne McKenzie at the Research School of Biological Sciences, The Australian National University, using the method of Greene *et al.*, (1978)) or Hha I or Sal I (Biolabs). Each digest was mixed with 6 µl of formamide solution, boiled for 1 min, cooled in iced water and loaded onto a 0.5 mm gel containing 4% acrylamide, 10.5 µg urea, 0.9 mls ammonium persulphate and 25 µl TEMED in TBE. The digests were electrophoresed at 30 mA until the marker dye reached the bottom of the gel (20 cm). The gel was then autoradiographed at -70°C overnight and processed in an Agfa-Gevaert developing machine.

The sizes of fragments were estimated as described in Chapter 5.

CHAPTER 3

SURVEYS FOR HONEY BEE VIRUSES

3.1 INTRODUCTION

Sacbrood virus (SBV) was thought to be uncommon in New South Wales (NSW) before 1976 (Hornitzky, 1982) but has since been reported to be the most common and widespread virus of honey bees in that state, as well as in South Australia (Dall, 1985; Hornitzky, 1982, 1984, 1985). Chronic bee paralysis virus (CBPV) has been frequently isolated in NSW and Victoria (Hornitzky, 1982, 1984; Reinganum, 1968), and Kashmir bee virus (KBV) has been detected in NSW (Hornitzky, 1981, 1982, 1984), South Australia (Bailey *et al.*, 1979; Dall, 1985), and Queensland (Rhodes, 1984; Rhodes and Teakle, 1978). Acute bee paralysis virus (ABPV), bee virus F (BVF), bee virus X (BVX), bee virus Y (BVY), black queen cell virus (BQCV), cloudy wing virus (CWV) and slow bee paralysis virus (SBPV) have also been detected in Australian honey bees (Bailey, 1981, 1982; Bailey *et al.*, 1979; Hornitzky, 1981, 1982, 1984; Reinganum, 1968) but their occurrence and distribution are not known. Little else is known of the frequency and distribution of viruses in Australian honey bees.

Much of the information given above has been published during the last 5 years, and hence was not known to me when my studies commenced. Therefore, I decided to begin my studies by surveying viruses in honey bee colonies in northern NSW and south-east Queensland during October 1981. A similar survey was done in south, central and far north coastal regions of Queensland during October 1982.

I also surveyed viruses in *Trigona* colonies in eastern NSW and south-east Queensland during February 1981 to determine whether they contained viruses similar to those of honey bees, as Bailey *et al.*, (1979) had suggested that KBV in Australian honey bees may have come from native *Trigona* species (Chapter 1).

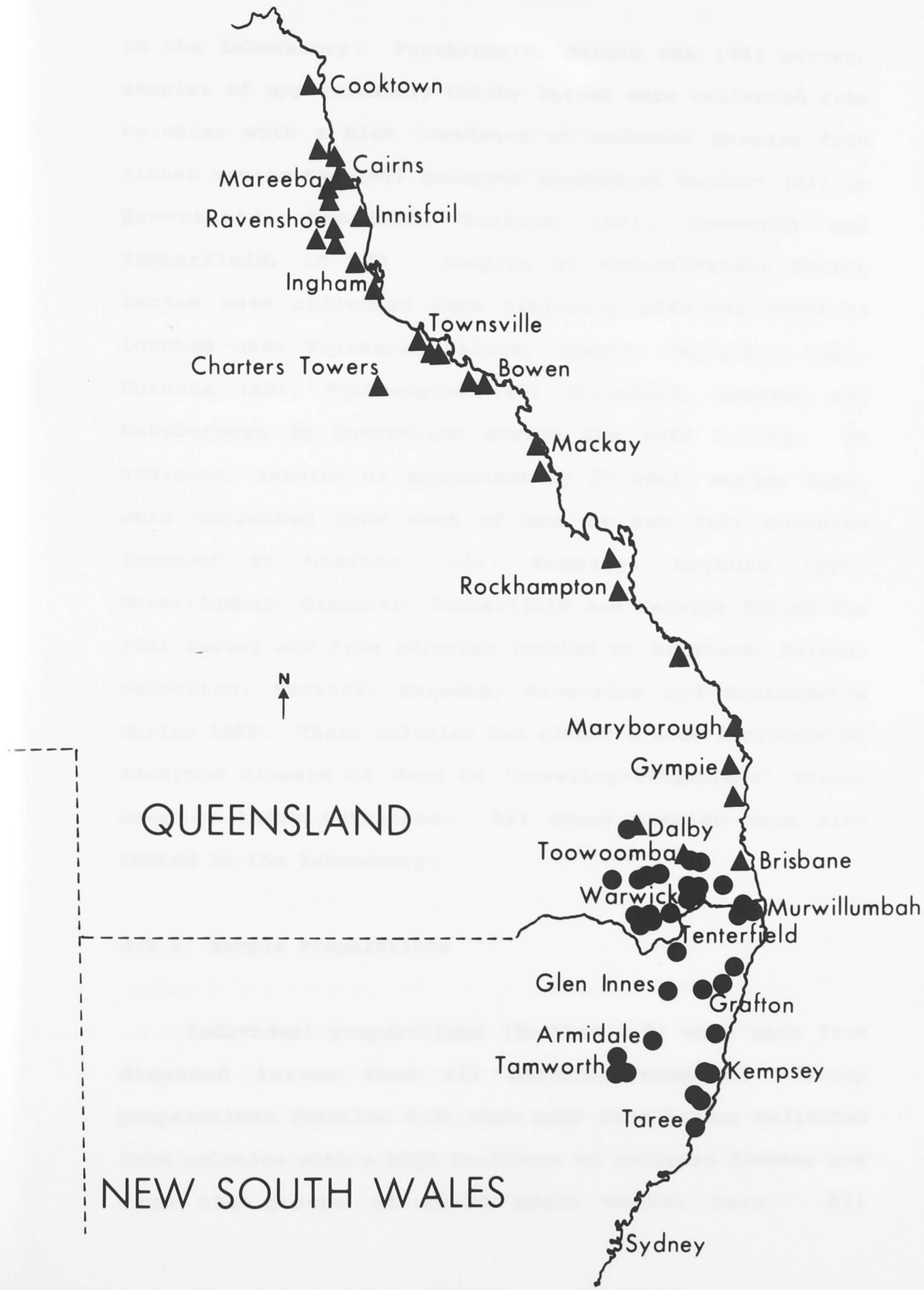
Some of the work reported in this chapter has been published in summarized form by Anderson and Gibbs (1982), and Anderson (1983).

3.2 SURVEYS OF VIRUSES IN HONEY BEE COLONIES

3.2.1 Sources of Specimens

Fifty-two honey bee colonies in northern NSW and south-east Queensland were inspected during October 1981. A further thirty were inspected in central and far north Queensland, mainly in coastal regions, during October 1982. The sampling localities are show in Figure 3.1. During both surveys, dead larvae were found in every hive inspected. Each was put into a small vial and later tested

FIGURE 3.1 The localities of the honey bee colonies sampled during the 1981 (circles) and 1982 (triangles) surveys. At least two colonies were examined and sampled at Armidale, Grafton, Kempsey, Leyburn, Tamworth, Taree and Tenterfield, during 1981.


Cooktown
Cairns
Mareeba
Ravenshoe
Innisfail
Ingham
Townsville
Charters Towers
Bowen
Mackay
Rockhampton
N
Maryborough
Gympie
QUEENSLAND
Dalby
Toowoomba
Brisbane
Warwick
Murwillumbah
Tenterfield
Glen Innes
Grafton
Armidale
Tamworth
Kempsey
Taree
NEW SOUTH WALES
Sydney

in the laboratory. Furthermore, during the 1981 survey, samples of approximately thirty larvae were collected from colonies with a high incidence of sacbrood disease from either one or two (x2) colonies located at Leyburn (x2) in Queensland, Armidale, Grafton (x2), Tamworth and Tenterfield, in NSW. Samples of approximately thirty larvae were collected from similarly affected colonies located near Brisbane, Cairns, Gympie, Herberton (x2), Kuranda (x2), Rockhampton (x2) Innisfail, Mareeba and Maryborough in Queensland during the 1982 survey. In addition, samples of approximately 30 adult worker bees, were collected from each of one or two (x2) colonies located at Grafton (x2), Kempsey, Leyburn (x2), Murwillumbah, Tamworth, Tenterfield and Warwick during the 1981 survey and from colonies located at Brisbane, Cairns, Herberton, Kuranda, Mareeba, Ravenshoe and Rockhampton during 1982. These colonies had either a high incidence of sacbrood disease or dead or 'crawling-flightless' worker bees at their entrances. All these samples were also tested in the laboratory.

3.2.2 Sample Preparations

Individual preparations (Section 2.3) were made from diseased larvae from all colonies sampled. Group preparations (Section 2.3) were made from larvae collected from colonies with a high incidence of sacbrood disease and from the groups of 20-30 adult worker bees. All

preparations were examined for virus particles by electron microscopy. They were also tested by gel diffusion using an antiserum prepared by me against CBPV, and antisera against the particles of BQCV, BVX, CWV, SBPV, KBV and SBV, and which was kindly supplied by Dr L. Bailey of Rothamsted Experimental Station, U.K. (Section 2.4). Some individuals from each locality shown in Figure 3.1 were kept frozen at -20°C for later use.

3.2.3 Bioassay

When attempting to inoculate apparently healthy white-eyed pupae with purified extracts from individuals collected during the 1981 survey, it was often found that the 'control pupae', namely those injected with PP buffer, produced KBV and SBV particles. This suggested that they were inapparently infected (see Chapter 4), and prevented their use for testing preparations in 1981 and 1982 by injection. However, an attempt was made during the 1982 survey to determine the distribution and frequency of occurrence of these inapparently infected pupae in honey bee colonies in the following way. Groups of twenty, apparently healthy, white-eyed pupae were removed from colonies located at Brisbane, Maryborough, Rockhampton and Mareeba, and individuals in each group were injected with sterile PP buffer. Injected pupae were put in small Petri dishes at room temperature for 4 days and transported on ice to the laboratory and stored at -20°C before testing.

3.2.4 Results

Virus particles were seen by electron microscopy only in those preparations which gave positive results in gel diffusion tests.

3.2.4.1 Individual Preparations

There was no evidence that larvae infected with SBV were simultaneously infected with any other virus; SBV was the only virus detected by serological tests in individual preparations. The tests showed that larvae infected with SBV were present in most colonies; 85% and 93% of the colonies were found to be infected with this virus in the 1981 and 1982 surveys respectively. SBV was isolated from larvae from at least one colony from every locality surveyed (Figure 3.1).

3.2.4.2 Group Preparations

Larvae infected with SBV and BQCV were found in the 7 colonies with severe sacbrood disease which were sampled in 1981. SBV infected larvae were found in all 12 severely affected colonies sampled in 1982; larvae infected with BQCV were detected in 11 of these colonies. Thus 13.5% and 36.7% respectively of colonies sampled in the 1981 and 1982 surveys contained larvae infected with BQCV.

Two of the 9, and 3 of the 7 group preparations from adult worker bees collected in the 1981 and 1982 surveys respectively contained CBPV. A further 4 of the 9 and 1 of the 7 contained SBV and one of the 7 contained CWV; none of the preparations contained mixtures of viruses. The colonies in both surveys which yielded workers infected with CBPV were also found to have a high incidence of sacbrood disease, and larvae infected with BQCV and SBV were isolated from them. Larvae infected with SBV and BQCV were also found in the colony which yielded workers infected with CWV. Furthermore, SBV particles were present in both larvae and adult worker bees collected from the same colony.

3.2.4.3 Inapparent Infections

SBV particles were detected in extracts of healthy white-eyed pupae collected from colonies at Brisbane, Maryborough, Rockhampton and Mareeba. Larvae showing symptoms of sacbrood were present in each of these colonies. No other inapparent virus infections were found in pupae from these localities.

3.3 SURVEY OF VIRUSES IN *TRIGONA* COLONIES

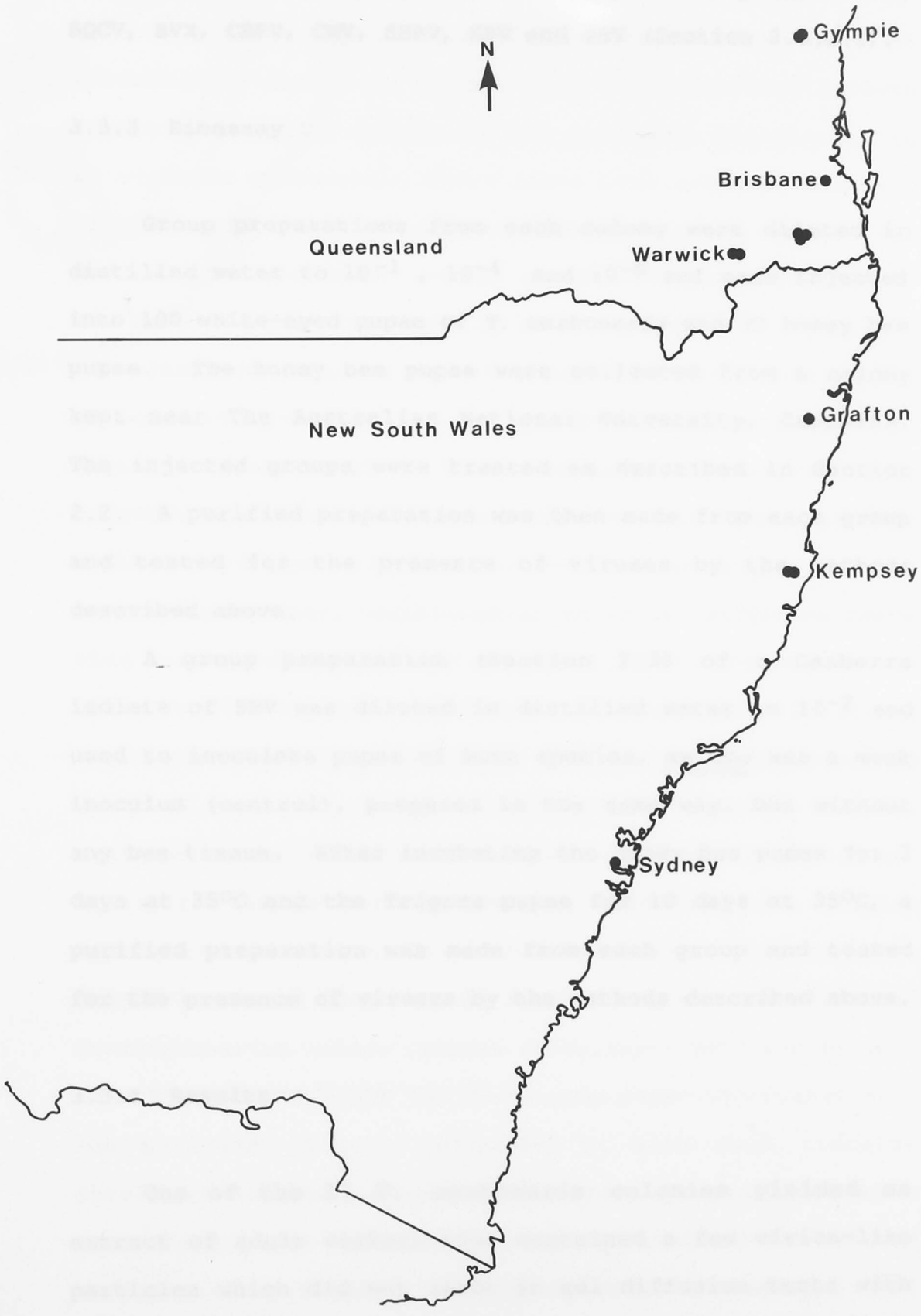
3.3.1 Sources of Specimens

Worker bees, larvae and pupae were collected from 11 colonies of *Trigona carbonaria* and from 1 colony of *T. australis* found in the coastal regions of northern NSW and southern Queensland (Figure 3.2); the *T. australis* colony was located at Warwick in Queensland. Comb from a *T. carbonaria* colony containing healthy young (white-eyed) pupae was also collected so that extracts of *Trigona* could be bioassayed by injecting them into pupae of the same species, a procedure commonly used for ~~testing~~ assaying for bee viruses (Bailey and Woods, 1977b). Worker bees from an unidentified species of *Trigona* found in the Kakadu National Park, Northern Territory, were kindly collected for me by Dr I. D. Naumann of the CSIRO Division of Entomology, Canberra.

3.3.2 Sample Preparation and Testing

Group preparations were prepared from worker bees from each colony as described in Section 2.3. They were examined for virus particles by negative staining electron microscopy. In addition, each extract was tested serologically for the presence of antigens of known bee viruses by gel diffusion tests using antisera, kindly

FIGURE 3.2 The localities of the *Trigona* colonies sampled during the 1981 survey.


N
Gympie
Brisbane
Queensland
Warwick
New South Wales
Grafton
Kempsey
Sydney

provided by Dr L. Bailey, prepared against the particles of BQCV, BVX, CBPV, CWV, SBPV, KBV and SBV (Section 2.5.2.1).

3.3.3 Bioassay

Group preparations from each colony were diluted in distilled water to 10^{-1}, 10^{-4} and 10^{-6} and each injected into 100 white-eyed pupae of *T. carbonaria* and 30 honey bee pupae. The honey bee pupae were collected from a colony kept near The Australian National University, Canberra. The injected groups were treated as described in Section 2.2. A purified preparation was then made from each group and tested for the presence of viruses by the methods described above.

A group preparation (Section 2.3) of a Canberra isolate of SBV was diluted in distilled water to 10^{-2} and used to inoculate pupae of both species, as too was a mock inoculum (control), prepared in the same way, but without any bee tissue. After incubating the honey bee pupae for 3 days at 35°C and the *Trigona* pupae for 10 days at 35°C, a purified preparation was made from each group and tested for the presence of viruses by the methods described above.

3.3.4 Results

One of the 11 *T. carbonaria* colonies yielded an extract of adult workers that contained a few virion-like particles which did not react in gel diffusion tests with

any of the 7 antisera. The particles were isometric, of uniform size and about 30 nm in diameter. Their size was estimated by mixing an aliquot with sap containing tobacco mosaic virus (TMV) particles and examining the mixture in an electron microscope; their sizes were compared directly on a contact print of an electron micrograph and it was assumed that the modal length of TMV particles is 300 nm. The TMV was kindly supplied by Dr A.J. Gibbs.

Purified extracts from 6 groups of *T. carbonaria* gave a band of precipitate in gel diffusion tests using antiserum prepared by Dr L. Bailey against CBPV particles. However, these bands were not observed when using a CBPV antiserum (reciprocal titre 64) prepared by me (see Section 2.4). Furthermore, results obtained in gel diffusion tests when using this freshly prepared CBPV antiserum, Dr Bailey's CBPV antisera, CBPV particles (purified in 10-40% sucrose gradients) and extracts from healthy honey bees and *Trigona*, confirmed that Dr Bailey's CBPV antiserum reacted with an antigen present in healthy bees and *Trigona*. Precipitin bands showing partial identity (i.e. giving 'spurs') were obtained in gel diffusion tests when extracts from *Trigona* and from healthy adult honey bees were tested, in neighbouring wells, against Dr Bailey's CBPV antiserum.

None of the honey bee or *Trigona* pupae inoculated with the purified *Trigona* extracts, or with mock inocula, developed disease symptoms. Purified preparations made from these pupae did not react in serological tests, nor were particles found in them by electron microscopy. The

honey bee pupae injected with SBV yielded preparations of SBV particles, as was shown by serological tests and by electron microscopy. However, extracts from similarly injected *Trigona* pupae did not react in serological tests, nor were particles seen in them by electron microscopy, indicating that SBV did not replicate in *Trigona*.

3.4 DISCUSSION

I found no evidence that *Trigona* is a source of viruses for honey bees in eastern Australia. It was surprising to find that virus infections in *Trigona* seem to be much less common than they are in honey bees; most honey bee colonies yield one or more of the 13 or so viruses that have been isolated from honey bees (Bailey *et al.*, 1981a). It is unlikely that the methods which successfully isolate honey bee viruses, and which were used for *Trigona*, are ineffective, as the same methods are effective for isolating viruses from *Bombus* species (Gibbs, personal comm.). Perhaps this result reflects a difference in selection pressures; a wild native species like *Trigona* may respond to pathogens by becoming genetically resistant, whereas a domesticated species is possibly selected by man for characters unlinked with disease resistance, and hence may be susceptible.

There was insufficient time during my studies for further work on the picornavirus-like particles seen in one *Trigona* extract, but it would be interesting to determine

whether these are related to the picornaviruses of honey bees. Nevertheless, this observation is consistent with the idea that the viruses of social animals are often picornaviruses, perhaps because these depend on close proximity between individual hosts in a population for transmission (e.g. by faecal contamination of food). The majority of honey bee viruses are picornaviruses, similar viruses are common in vertebrates and one has even been isolated from Australian termites (Gibbs *et al.*, 1970).

It was surprising that, of the 7 viruses for which I tested, only 4 were found in the two extensive surveys of honey bee colonies in eastern Australia. Perhaps more would have been found if I had sampled at different times of the year. Alternatively, the other 3 viruses for which I tested may be uncommon in the areas sampled.

Both surveys showed that SBV was the most common virus in honey bee colonies in eastern Australia in the spring of 1981 and 1982. This result is interesting because Hornitzky (1982), reported that sacbrood disease was virtually unknown in NSW prior to 1976. It is possible that a particularly virulent SBV strain arose or was imported some time before 1981 or, alternatively, that the virus may have been present previously, but was not recognized. To investigate these possibilities, it seemed worthwhile to study strain variation of SBV in some detail. This work is reported in Chapter 5.

Rhodes and Teakle (1978) described symptoms of diseased larvae from south-east Queensland which, they

claimed, were caused by KBV. These symptoms (described in Chapter 1) are similar to those of European foulbrood (EFB) disease in honey bee larvae which is caused by the bacterium *Melissococcus pluton*. Some doubt exists as to whether the symptoms reported by Rhodes and Teakle were caused by KBV, as their attempts to infect healthy larvae with bacteria-free extracts from larvae with 'KBV disease' were unsuccessful because all the larvae fed these extracts were removed from the colony by worker bees within 48 hours; that is, Koch's Postulates were not fulfilled. It is noteworthy that during the 1981 survey I found many dead larvae with symptoms similar to those described by Rhodes and Teakle in colonies in south-east Queensland. However, no virus particles were seen in extracts purified from these larvae, nor did the extracts react in gel diffusion tests using antisera prepared against the particles of several honey bee viruses, including KBV. I did not examine these larvae for *M. pluton* and their cause of death remains unknown.

A significant finding of the 1981 and 1982 surveys was that healthy white-eyed honey bee pupae appeared to be inapparently infected with viruses. Bailey *et al.*, (1981a) found SBV, BQCV, and sometimes CBPV particles in immature bees (pre-pupae and pupae) in nature, but did not report whether the pupae from which they were isolated appeared healthy. Therefore, a study to check whether pupae were inapparently infected with bee viruses seemed warranted because white-eyed pupae are often used by insect

pathologists to propagate bee viruses. This study is reported in the following chapter.

CHAPTER 4

INAPPARENT VIRUS INFECTIONS OF HONEY BEE PUPAE

4.1 INTRODUCTION

When susceptible cells are infected by virus particles, they usually become acutely infected, rapidly produce large numbers of virus particles and die (a cytocidal response) or, alternatively, they may not die but recover and become persistently infected and yield smaller numbers of particles over long periods of time. Infections of the latter type may persist as 'inapparent' infections in the host cells for many generations producing few or no detectable symptoms but possessing the 'latent' capacity to yield particles and initiate a cytocidal infection and response.

Words such as 'inapparent' or 'latent', when used to define infections, are often used ambiguously. I use 'inapparent' to describe infections where the virus cannot be directly detected before being activated, and where there is no knowledge of the state of that virus. By contrast, I use 'latent' to describe inapparent infections in which the virus genome is in a state or site that is not involved in acute infections.

4.1.1 Latent Virus Infections

A well-known type of latent virus infection is the virus-host relationship called lysogeny, involving viruses of bacteria (bacteriophages). In this relationship, the genome of an infecting bacteriophage becomes part of the bacterial genome and hence reproduces in synchrony with it. Expression of most of the genes of the prophage, or provirus, is suppressed by a repressor produced by a small part of the prophage genome, and the infected bacterium undergoes normal cell division to produce lysogenic progeny. The lysogenized bacteria are immune to infection by the phage whose genome they contain. In some however, expression of the prophage genome may be spontaneously derepressed, or activated, to initiate a cycle in which the bacterium produces large numbers of virus particles, lyses, and liberates mature virions which may infect a susceptible bacterium and give rise to either a cytocidal or to a lysogenic infection (Hershey and Dove, 1971).

Lysogeny is not known to occur in plant-virus relationships and, in animal-virus relationships, has only been proven among the retrovirus group (Fenner *et al.*, 1974).

4.1.2 Inapparent Virus Infections

Inapparent virus infections are common in plants and animals, particularly in insects (Bailey and Woods, 1974;

Brun and Plus, 1980; Evans and Harrap, 1982; Reinganum *et al.*, 1970). They have even been reported in insect cell cultures (Brun and Plus, 1980; Buckley, 1971; Davey and Dalgarno, 1974). Some inapparent virus infections of insects have been discovered after the infections were 'stimulated' or 'activated' to become cytocidal. The methods used to activate inapparent virus infections in insects have included crowding, incubation at high and low temperatures, injection of foreign proteins, foreign viruses, milk fractions, rabbit sera and microsporidian spores, as well as the use of vibration (Aruga and Hukuhara, 1960; Aruga *et al.*, 1963; Bailey and Gibbs 1964; Evans and Harrap, 1982; Smith, 1967). The mechanisms by which inapparent insect virus infections become activated are not known.

There are few reports on how inapparent virus infections become established in insects. Jousset and Plus (1975) reported that some inapparent picorna-like virus infections are transmitted in the eggs of *Drosophila*, and Smirnoff (1962) reported that inapparent nuclear polyhedrosis virus (NPV) infections may be established in larvae of the sawfly, *Neodiprion swainei*, by feeding small numbers of NPV particles to older larval instars.

4.1.3 Inapparent Virus Infections of the Honey Bee

At the beginning of my studies, inapparent infections of acute bee paralysis virus (ABPV), black queen cell virus

(BQCV), and sacbrood virus (SBV) had been reported in adult worker bees (Bailey, 1967; Bailey and Fernando, 1972; Bailey and Gibbs, 1964; Bailey and Woods, 1974; Bailey *et al.*, 1963; Bailey *et al.*, 1981a). Bailey and Gibbs (1964) reported ABPV as a common inapparent infection of both adult worker honey bees and pupae. More recently, Dall (1985) reported inapparent infections of Kashmir bee virus (KBV) and SBV in bee pupae in Australia; this study was done independently of, but concurrently with, the work reported in this thesis, and its findings will be discussed where appropriate throughout the text of this chapter. The work reported here has been presented at two conferences (Anderson, 1985; Anderson and Gibbs, 1984).

4.1.4 The Studies Reported in this Chapter

Most bee viruses have traditionally been propagated in bee pupae (Bailey and Woods, 1977b) because attempts to cultivate bee viruses in hosts other than honey bees or in susceptible cultured cells have been unsuccessful (Bailey and Gibbs, 1964; see Chapter 1), and because of the ease with which bee pupae may be handled. However, preliminary experiments done at the beginning of my studies during the surveys (Chapter 3), indicated that bee pupae often became diseased and yielded KBV or SBV after being injected either with sterile 10 mM potassium phosphate buffer (PP buffer) or extracts made by grinding apparently healthy bee pupae in PP buffer. These results suggested that apparently

healthy pupae were frequently infected inapparently, and also that such infections could be activated merely by injecting PP buffer. It seemed worthwhile therefore to test whether methods could be developed for cultivating individual bee viruses in pupae despite the presence of potentially contaminating inapparent infections.

This chapter reports a study of inapparent virus infections in honey bee pupae. Investigations to determine their presence, occurrence, suppression and establishment in pupae are described.

4.2 DETERMINING THE PRESENCE OF INAPPARENT VIRUS INFECTIONS IN BEE PUPAE

4.2.1 Sources of Materials

Bee pupae used in the following experiments were obtained from normal apparently healthy honey bee colonies located in the Australian Capital Territory (ACT).

4.2.2 Statistical Analyses

Statistical models from the statistical package GENSTAT V (Release 4.04B, Copyright 1984, Lawes Agricultural Trust, Rothamsted Experimental Station) were used to analyse some of the experimental results.

4.2.3 The Reported Experiments

The experimental strategy was first to determine whether bee pupae became acutely infected with KBV or SBV after being inoculated with PP buffer and held at different temperatures. Other experiments were then done to test whether the pupae which became infected by this method did so because of the activation of inapparent infections.

4.2.4 Experiment 1

The aim of this experiment was to determine whether virus particles could be detected in white-eyed pupae after they had been injected with sterilized PP buffer and incubated at 30°C or at 35°C.

4.2.4.1 Methods

A frame of wax comb consisting of empty cells was placed in the centre of a normal bee colony and, within 3 days, most of the cells contained eggs which later hatched into larvae and developed normally. Three days after the brood cells were capped, the frame was taken from the colony to the laboratory where two sections of comb, one containing 35 capped cells, the other 50 capped cells, were sliced out of the frame and kept at 30°C and 35°C respectively, for 3 days. A further 300 white-eyed pupae

were removed from the remainder of the comb using sterile techniques, and separated at random into ten groups, each of 30 individuals. Into each individual of four of the groups was injected 10 µl of sterilized PP buffer; two of these groups were kept at 30°C and the other two at 35°C, both for 3 days. Individual pupae in another four groups were not injected, and again two of these groups were held at 30°C for 3 days and the other two at 35°C for 3 days. The remaining two groups of pupae were tested for virus immediately; an extract was prepared from each individual pupa, and later from each incubated individual, and tested for the presence of ABPV, BQCV, bee virus X (BVX), CBPV, cricket paralysis virus (CrPV), cloudy wing virus (CWV), KBV and SBV particles using gel diffusion serological tests and negative staining electron microscopy tests, as described in Section 2.5. Extracts from those pupae which had not been injected and incubated were also tested for the presence of KBV and SBV particles using indirect ELISA tests, as described in Section 2.5.2.2.

4.2.4.2 Results

4.2.4.2.1 Pupae Injected with PP Buffer

Table 4.1 shows the number of virus-infected and healthy pupae within each injected group, as determined by the gel diffusion tests. These results show that some

TABLE 4.1 The number of pupae yielding virus particles, after injection with PP buffer and incubation at 30°C or 35°C (*).

INCUBATION TEMPERATURE (°C)	# OF PUPAE YIELDING: KBV	SBV(+)	# OF HEALTHY PUPAE
30	4	1	25
30 (replica)	2	2	26
35	9	6	15
35 (replica)	10	5	15

(*): See text for experimental details.

(+): KBV = Kashmir bee virus; SBV = sacbrood virus.

pupae became infected with KBV and SBV when injected with PP buffer and incubated at 30°C and at 35°C.

Cross-tabulated tables of counts, such as those given in Table 4.1, are referred to as contingency tables and various hypotheses concerning these tables can be examined using simple χ^2 goodness-of-fit tests. A more general and flexible procedure for analysing contingency tables is to fit log-linear regression models; this assumes that the counts are independently drawn from a Poisson distribution. Such analyses produce statistics that are analogous to those given by the simpler goodness-of-fit statistics. The hypotheses of interest for the data of Table 4.1 are:

(1) Are the proportions of infected pupae independent of temperature? Inspection of Table 4.1 clearly shows that a higher proportion of pupae became infected at 35°C than at 30°C. The log-linear analysis produced a χ^2 statistic of 17.44 with 1 degree of freedom (d.f.), which is significant at the 0.01% level of probability.

(2) Are the proportions of pupae with KBV or SBV infections independent of temperature? Again inspection of Table 4.1 shows that these proportions are independent of temperature; for instance, the proportion of KBV infected pupae at 30°C (0.1) is almost equal to the proportion of SBV infected pupae at this temperature (0.05), and similar results were obtained at 35°C. The χ^2 statistic for this hypothesis is 0.03 with 1 d.f., which is not significant at the 5% level of probability. Nevertheless, the data shown in Table 4.1 indicates little variability between the

numbers of SBV and KBV infected pupae in the replica experiments.

4.2.4.2.2 Pupae Not Injected with PP Buffer

No virus antigens were detected by gel diffusion tests in those extracts prepared from pupae immediately after they were extracted from their wax cells (i.e. from non-injected and non-incubated pupae). Similar results were obtained using the ELISA method despite its much greater sensitivity (Anderson, 1984).

Table 4.2 shows the number of virus infected and healthy pupae in groups held at 30°C and 35°C, detected using gel diffusion. The log-linear analysis showed that the higher incidence of SBV infected pupae at 35°C was statistically significant ($\chi^2=10.24$, d.f.=1, $p<0.01$).

Interestingly, no pupa was found to be infected with more than one virus; some extracts reacted with KBV antiserum, others with SBV antiserum, but none with both.

4.2.4.3 Discussion

The results of these experiments showed that no virus could be detected, using gel diffusion or ELISA tests, in extracts obtained directly from apparently healthy pupae in a normal colony. However, SBV and KBV particles were found in apparently healthy pupae after they had been extracted (using sterilized forceps) from sealed cells of brood and

TABLE 4.2 The number of healthy or virus yielding pupae in uninjected groups incubated at 30°C or 35°C (*).

TREATMENT	INCUBATION TEMPERATURE	# OF PUPAE YIELDING: KBV	SBV(+)	# OF HEALTHY PUPAE
NI@	30	0	0	30
NI (replica)	30	0	0	30
NI and left in cells	30	0	0	35
NI	35	0	2	28
NI (replica)	35	0	2	28
NI and left in cells	35	0	4	46

(*): See text for experimental detail.

(+): SBV = sacbrood virus; KBV = Kashmir bee virus.

@: NI = not injected.

injected with sterilized PP buffer. It was also found that a small number of pupae became infected with SBV when kept in their capped cells at the higher temperatures. Dall (1985) also reported that inapparent SBV infections became activated in uninjected bee pupae used as experimental controls.

These results did not show whether pupae became infected by activation of inapparent infections, or from virus particles on the surface of pupae. Such contaminating particles may have infected the pupae either through grazes or wounds, such as broken hairs in the exoskeleton, or by contaminating the needle used for injection. The following experiment was done to test whether surface contamination was the source of infection.

4.2.5 Experiment 2

The aim of this experiment was to test whether the infections of KBV or SBV, which occurred in pupae after injection with sterilized PP buffer, resulted from KBV or SBV particles on the surface of some pupae.

4.2.5.1 Methods

One hundred and twenty white-eyed pupae were obtained as described in Experiment 1 (Section 4.2.4.1). Thirty were inoculated with 10 µl of sterile PP buffer; another thirty were punctured through a dorso-lateral abdominal

intersegmental membrane with a sterile (new) gauge 30 stainless steel needle but no extract was injected; another thirty were washed in 5.0 ml of sterilized PP buffer, then 10 µl of the wash was injected into each of the remaining thirty pupae. All pupae were incubated at 35°C for 3 days, then an extract was obtained from each and tested for the presence of virus particles using gel diffusion tests as described in Section 4.2.4.1.

4.2.5.2 Results and Conclusions

KBV and SBV particles were each found in 5 pupae injected with the PP buffer, and in 5 pupae injected with the wash from thirty pupae. Two of the thirty extracts from pupae punctured with a sterile needle contained SBV particles. No other virus antigens were found in the extracts and, as before, there was no evidence for the presence of more than one virus in each pupa.

The results show no evidence that the KBV and SBV infections, which occur in pupae after injection with sterilized PP buffer, are caused by virus particles from the surface of pupae, nor is there evidence that activation results merely from the trauma of injection. However, not all infections may have resulted from activation because the same needle was used to inject all the pupae in a group. Hence, the needle may have become contaminated after piercing the exoskeleton of an inapparently infected pupa, thereby infecting the next injected pupa. The

likelihood of infections occurring by this method was tested in the following experiment.

4.2.6 Experiment 3

4.2.6.1 Methods

One hundred white-eyed pupae, obtained by the methods described in Experiment 1 (Section 4.2.4.1), were each injected with 10 µl of sterilized PP buffer, numbered serially, placed on filter paper inside a Petri dish, and incubated for 3 days at 35°C. An extract was then obtained from each pupa and tested for the presence of virus particles as described in Experiment 1. The results were analysed statistically to determine whether there were more 'runs' of infected individuals in the series than expected by chance. This was done by the method of van der Plank (1946) by recording the number of adjacent pairs of infected individuals in the series and comparing these with the number expected for Poissonian (random) events using the formula:

$$p = d(d-1)/n.$$

Where:

p = the expected number of pairs of adjacent diseased individuals if at random,

d = the number of diseased individuals observed in the series and,

n = the total number of individuals in the series.

When n is large, the standard error of the estimate is $\sqrt{p}$.

4.2.6.2 Results and Discussion

In one series of 100 injected pupae, 26 individuals became infected with KBV but only one pair was obtained. This compares with the predicted 6.5 pairs. Therefore, because n is large (100), 1 pair is more than 2 standard errors ($\sqrt{6.5} = 2.55$) from expected 6.5 pairs. In the same experiment 21 individuals became infected with SBV and, of these, no pairs were obtained, compared with the predicted 4.2 pairs. Thus there was no statistically significant evidence that the needle used to inject the PP buffer became contaminated after injecting an inapparently infected pupa and subsequently transferred the virus to other pupae of the same experimental group.

Preliminary attempts were made to locate the possible sites of inapparent infections in pupae by dissecting and removing the intact gut from pupae obtained from a colony where most, if not all, pupae were inapparently infected. Twenty guts were each mashed in 0.3 ml of PP buffer, and the extracts clarified by centrifuging in an Eppendorf bench centrifuge at 15,000 r.p.m. for 1 minute. The supernatant extracts were each tested for KBV and SBV virus particles by ELISA tests, as described in Chapter 2. In addition, ultra-thin sections were obtained from five other

guts, by the methods described by Lee and Furgala (1967a), and examined for virus particles in the electron microscope. No evidence of virus were obtained in any of these tests so the studies were not pursued any further. However, attempts were made to check whether pupae, inapparently infected with KBV or SBV, developed into inapparently infected workers and whether the infections in these workers could be activated in a similar way to those in pupae. These studies, which are of importance in an understanding of the ecology of these viruses, are described in the following section.

4.3 TESTING FOR INAPPARENT VIRUS INFECTIONS IN NEWLY EMERGED WORKER HONEY BEES

4.3.1 Methods

A comb of brood cells containing young white-eyed pupae was obtained from a colony in the ACT by the methods described in Section 4.2.4.1. Thirty white-eyed pupae were removed from their cells, each injected with 10 µl of sterilized PP buffer and kept at 35°C for 3 days. The comb was returned immediately to the parent colony until the emergence of young worker bees was imminent. It was then removed to the laboratory and a section of comb containing 84 cells of sealed brood was sliced out and kept at 35°C. As the worker bees emerged from their cells they were captured and injected with 10 µl of sterilized PP buffer

and caged at 35°C. Worker bees were injected in the same way as pupae (Section 2.2) except they were anaesthetised with carbon dioxide before injection. Caged bees that died were frozen at -20°C until the completion of the experiment (7 days after inoculation), when an extract was obtained from each, and from each of the remaining live bees in the cages. These were tested for the presence of virus particles as described in Section 4.2.4.1. An extract was also obtained from each of the thirty pupae initially removed from the comb and each was similarly tested for the presence of virus particles.

4.3.2 Results and Conclusions

KBV and SBV particles were found in 4 (13%) and 5 (17%) of the extracts from pupae, and in 10 (12%) and 16 (19%) of the 84 extracts from worker bees. Thus the incidence of the two viruses recovered from pupae was almost identical to that in their adult sisters. In no instances were mixed infections detected.

The results show that young adult worker bees may be inapparently infected with KBV and SBV when they emerge from their cells and these infections may be activated in just the same way as they are in pupae.

The studies described above had shown that sterilized PP buffer activated inapparent infections in pupae and worker bees. It thus seemed worthwhile to test other

solutions for their ability to activate inapparent virus infections. These studies are reported in the following section.

4.4 TESTING SOLUTIONS FOR THEIR ABILITY TO ACTIVATE INAPPARENT VIRUS INFECTIONS IN BEE PUPAE

4.4.1 Methods

Two hundred and eighty white-eyed pupae were obtained by the method described in Section 4.2.4.1, and distributed at random into fourteen groups of 20 individuals. Every pupa in a pair of groups received one or other of the following treatments; an injection of 10 µl of either 10 mM PP buffer, pH 6.7, or 50 mM potassium phosphate buffer, pH 6.7, or 35 mM borate buffer, pH 7.0, or Insect Ringers solution, pH 7.0 (156 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$), or 10 mM Tris, pH 7.0, or 10 mM ammonium acetate, pH 7.0, or pre-immune rabbit serum, or distilled water. All inocula, except the rabbit serum, were sterilized before being injected. A sterile 30 gauge stainless steel needle was used to inject each solution. Injected pupae were kept for 3 days at 35°C, then an extract was obtained from each and tested for the presence of virus particles using ELISA and gel diffusion tests as described in Section 4.2.4.1.

4.4.2 Results and Discussion

The numbers of healthy and virus infected pupae within each group, detected using gel diffusion tests, are shown in Table 4.3. Identical results were obtained using the more sensitive ELISA method, indicating that the gel diffusion method was adequately sensitive for detecting the presence of activated virus particles (i.e. pupae were either infected or healthy and there were no intermediate concentrations of virus). Thus, in subsequent experiments the gel diffusion method was used to detect the presence of activated virus particles because it was the simpler technique.

The results in Table 4.3 show that inapparent infections of BQCV and CrPV were activated in some pupae; neither had previously been detected. It was surprising to find bee pupae inapparently infected with CrPV. This virus was originally isolated from Australian crickets and has since been propagated in many insects (Scotti *et al.*, 1981; Wigley and Scotti, 1983), nevertheless, the virus has not been previously reported in bee colonies in nature.

The proportion of infected pupae from Table 4.3 are given in Table 4.4. Also given are the approximate 95% confidence limits for these proportions, which were obtained by fitting a linear logistic regression model from the GENSTAT package (Section 4.2.2) to the data. The

TABLE 4.3 The number of pupae from groups of 20 which yielded virus when injected with various solutions (*).

SOLUTION INJECTED	# OF PUPAE YIELDING: SBV	KBV	BQCV	CrPV(+)	TOTAL # OF INFECTED PUPAE
(A) 10 mM PP buffer	3	6	0	0	9
replicate	4	4	0	0	8
(B) 50 mM PP buffer	1	7	1	0	9
replicate	2	5	1	0	8
(C) borate buffer	6	6	0	1	13
replicate	4	3	0	2	9
(D) Insect Ringers	3	4	0	0	7
replicate	2	3	0	0	5
(E) Tris buffer	5	2	1	0	8
replicate	4	5	1	0	10
(F) ammon. acetate	4	6	0	1	11
replicate	6	6	0	0	12
(G) rabbit serum	3	5	1	0	9
replicate	3	4	1	0	8
(H) dist. water	2	2	0	0	4
replicate	2	1	1	0	4

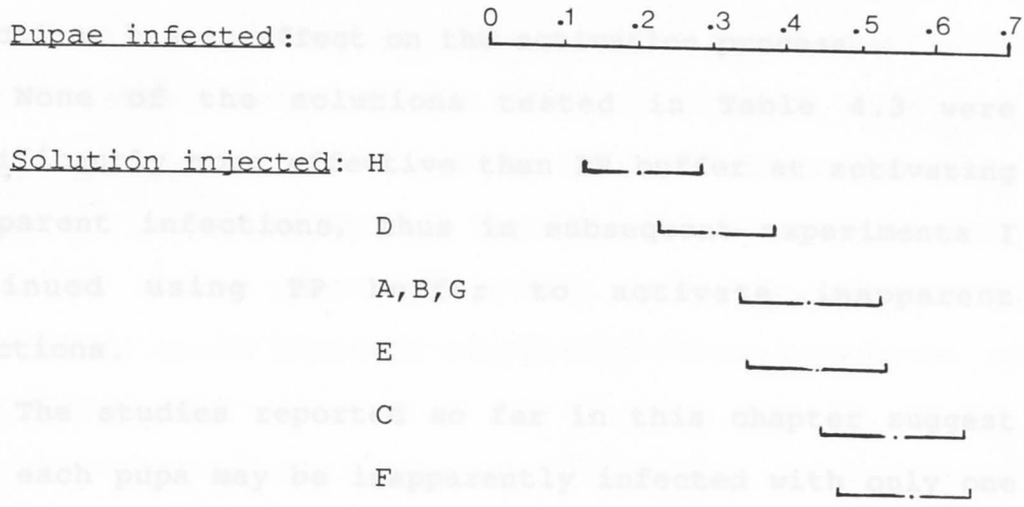
(*): See text for experimental details.
(+): SBV = sacbrood virus; KBV = Kashmir bee virus; BQCV = black queen cell virus; CrPV = cricket paralysis virus.

TABLE 4.4 The proportions of pupae showing inapparent virus infections after injection with various solutions, together with the approximate upper and lower 95% confidence limits (*).

SOLUTION INJECTED(+)	PROPORTION OF PUPAE INFECTED(@)	95% CONFIDENCE LIMITS: LOWER	UPPER
A	.425	.334	.521
B	.425	.334	.521
C	.550	.454	.643
D	.300	.226	.387
E	.450	.358	.546
F	.575	.479	.666
G	.425	.334	.521
H	.200	.134	.287

(*): See text for experimental details.
(+): See Table 4.3 for details of solutions injected.
(@): Obtained from total of infected pupae in replica groups in Table 4.3.

results in Table 4.4 can be represented diagramatically as follows.



The overlap of the upper and lower confidence limits obtained with different treatments indicates the considerable similarity between the efficiency of the solutions to activate inapparent virus infections in bee pupae. However, distilled water (solution H) activated significantly fewer inapparent virus infections than the other solutions, except for Insects Ringers (solution D).

The results of these experiments clearly show that solutions other than PP buffer, can activate inapparent virus infections when injected into inapparently infected pupae. Dall (1985) also used rabbit sera to activate inapparent SBV and KBV infections in bee pupae but reported that sera from some rabbits activated more efficiently than others.

There was some evidence in my studies that solutions containing salts were more effective than distilled water at activating inapparent virus infections, suggesting that perhaps the osmotic environment created in a pupa after injection, has an effect on the activation process.

None of the solutions tested in Table 4.3 were significantly more effective than PP buffer at activating inapparent infections, thus in subsequent experiments I continued using PP buffer to activate inapparent infections.

The studies reported so far in this chapter suggest that each pupa may be inapparently infected with only one virus; no extract of a single pupa ever contained more than one virus. This was most unexpected because in some experiments enough pupae were tested to obtain significant numbers of doubly infected pupae if infection with different viruses was independent. For example, in the experiment reported in Table 4.3, 17% of the 320 pupae tested were infected with SBV, and 21% with KBV, thus 11 would be expected to be doubly infected (17% of 21% of 320). Thus, it seemed worthwhile to test whether individual pupae were inapparently infected with only one virus. These studies are reported in the following section.

4.5 SUPPRESSING INAPPARENT VIRUS INFECTIONS IN BEE PUPAE

Bailey *et al.*, (1964) found that SBV particles mixed with SBV antiserum did not cause sacbrood disease in young bee larvae, and Bailey and Gibbs (1964) found that adult bees injected with dilute ABPV antiserum were not susceptible to concentrations of ABPV particles that were lethal to untreated bees. Therefore, I decided to test whether PP buffer mixed with rabbit sera prepared against bee viruses could suppress inapparent virus infections in honey bee pupae.

4.5.1 Experimental Strategy

Because there was some evidence from the activation experiments that inapparent KBV infections may be more common than inapparent SBV infections, I decided first to inject groups of 20 pupae with two-fold dilutions of KBV antiserum in an attempt to suppress inapparent KBV infections. From these results, a dilution of KBV antiserum that did not harm pupae but suppressed inapparent KBV infections was chosen and used with two-fold dilutions of SBV antiserum to attempt to suppress inapparent SBV infections in pupae. Likewise, an appropriate dilution of SBV antiserum was chosen and mixed with the appropriate dilution of KBV antiserum and used with two-fold dilutions of BQCV antiserum in an attempt to suppress inapparent BQCV infections.

4.5.2 The Experiments

White-eyed pupae used in these experiments were obtained from a normal healthy colony in the ACT as described 4.2.4.1.

4.5.2.1 Experiment 1

Two-fold serial dilutions of KBV antiserum in PP buffer were injected into groups of 20 white-eyed pupae. The pupae were incubated at 35°C for 3 days, then an extract was obtained from each pupa and tested for the presence of bee viruses using gel diffusion tests, as described in Section 4.2.4.1.

4.5.2.2 Experiment 2

Two-fold serial dilutions of SBV antiserum in PP buffer, each containing a 1:16 dilution of KBV antiserum, were injected into groups of 20 white-eyed pupae. Injected pupae were treated as described in the previous experiment.

4.5.2.3 Experiment 3

Two-fold serial dilutions of BQCV antiserum in PP buffer, each containing 1:16 dilutions of KBV and SBV antisera, were injected into groups of 20 white-eyed pupae.

Injected pupae were treated as described in Section 4.5.2.1.

4.5.2.4 Results and Conclusions

The percentages of healthy and virus infected pupae in each injected group of 20 pupae, for Experiments 1, 2 and 3, are shown in Figure 4.1. These show that inapparent BQCV, KBV and SBV infections in pupae could all be suppressed by injecting inapparently infected individuals with specific antisera diluted to 1:128. However a 1:16 dilution of specific antiserum effectively suppressed each inapparent infection and did not harm the pupae and was therefore used in all subsequent experiments.

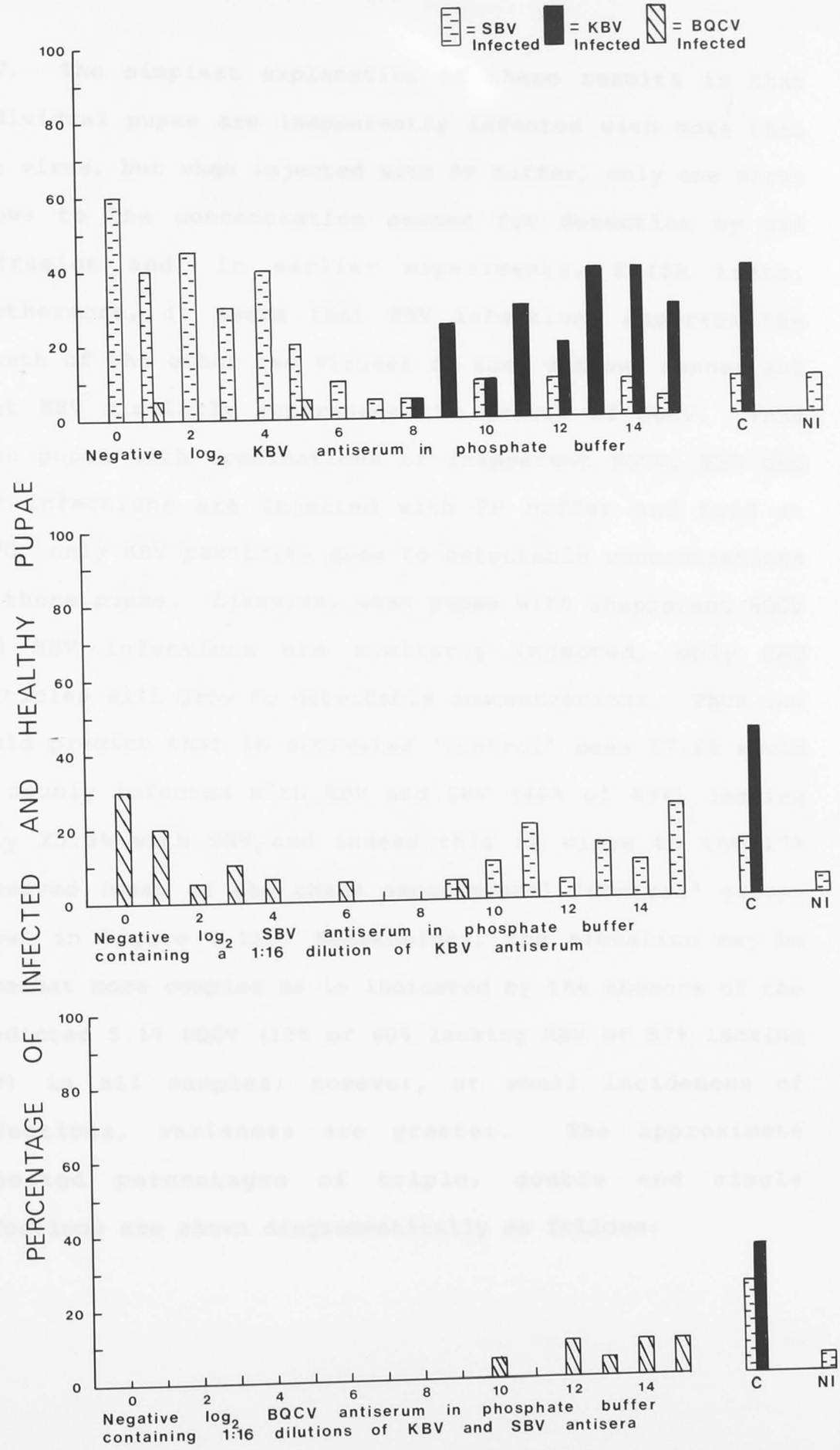
As before, only one virus was found in the extract of each individual, but the results show that more SBV infections (approx. 40%) were found in groups in which inapparent KBV infections were suppressed than were found in groups in which those infections were not suppressed (10%). Similarly, more pupae were found to be infected with BQCV (approx. 15%) in groups in which inapparent KBV and SBV infections were suppressed than were found in groups in which those infections were not suppressed (approx. 5%) (Figure 4.1).

When appropriately suppressed by antisera (i.e. with dilutions less than 1:16) it was clear that about 43% of the pupae carried activatable SBV, similarly about 15% carried activatable BQCV and about 40% carried activatable

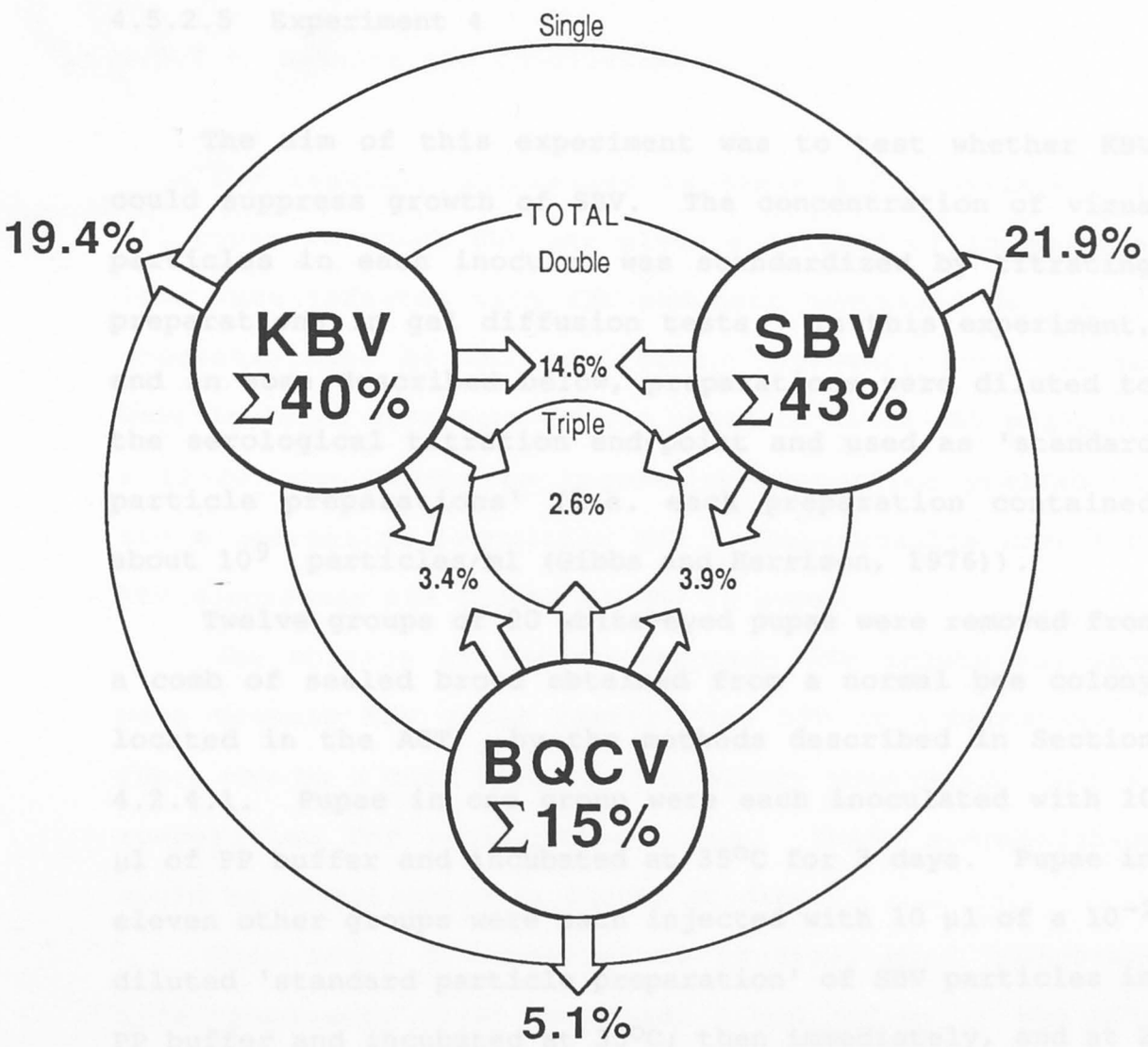
FIGURE 4.1 The percentages of pupae with inapparent virus infections (20 tested) after injection with dilutions of Kashmir bee virus (KBV) antiserum (top), dilutions of sacbrood virus (SBV) antiserum containing a 1:16 dilution of KBV antiserum (middle), and dilutions of black queen cell virus (BQCV) antiserum containing 1:16 dilutions of both KBV and SBV antisera (bottom).

NI : Not injected pupae.

C : Pupae injected with 10mM potassium phosphate buffer, pH 6.7 ('controls').


= SBV Infected
= KBV Infected
= BQCV Infected
PERCENTAGE OF INFECTED AND HEALTHY PUPAE
100
80
60
40
20
0
0 2 4 6 8 10 12 14
C NI
Negative log2 KBV antiserum in phosphate buffer
Negative log2 SBV antiserum in phosphate buffer containing a 1:16 dilution of KBV antiserum
Negative log2 BQCV antiserum in phosphate buffer containing 1:16 dilutions of KBV and SBV antisera

KBV. The simplest explanation of these results is that individual pupae are inapparently infected with more than one virus, but when injected with PP buffer, only one virus grows to the concentration needed for detection by gel diffusion and, in earlier experiments, ELISA tests. Furthermore, it seems that KBV infections suppress the growth of the other two viruses in some unknown manner and that SBV similarly suppresses the growth of BQCV. Thus when pupae with combinations of inapparent BQCV, KBV and SBV infections are injected with PP buffer and held at 35^{o}C, only KBV particles grow to detectable concentrations in those pupae. Likewise, when pupae with inapparent BQCV and SBV infections are similarly injected, only SBV particles will grow to detectable concentrations. Thus one would predict that in activated 'control' bees 17.2% would be doubly infected with KBV and SBV (40% of 43%) leaving only 25.8% with SBV, and indeed this is close to the 17% observed (mean of the three experimental 'control' groups shown in Figure 4.1). Nonetheless, the situation may be somewhat more complex as is indicated by the absence of the predicted 5.1% BQCV (15% of 60% lacking KBV of 57% lacking SBV) in all samples; however, at small incidences of infections, variances are greater. The approximate expected percentages of triple, double and single infections are shown diagrammatically as follows:


Infections
Single
TOTAL
Double
Triple
19.4%
21.9%
KBV
Σ40%
SBV
Σ43%
14.6%
2.6%
3.4%
3.9%
BQCV
Σ15%
5.1%

One simple and testable prediction of these conclusions is that KBV suppresses the growth of SBV. This was tested in the following experiment.

4.5.2.5 Experiment 4

The aim of this experiment was to test whether KBV could suppress growth of SBV. The concentration of virus particles in each inoculum was standardized by titrating preparations in gel diffusion tests. In this experiment, and in some described below, preparations were diluted to the serological titration end-point and used as 'standard particle preparations' (i.e. each preparation contained about 10^9 particles/ml (Gibbs and Harrison, 1976)).

Twelve groups of 20 white-eyed pupae were removed from a comb of sealed brood obtained from a normal bee colony located in the ACT by the methods described in Section 4.2.4.1. Pupae in one group were each inoculated with 10 µl of PP buffer and incubated at 35°C for 3 days. Pupae in eleven other groups were each injected with 10 µl of a 10^{-2} diluted 'standard particle preparation' of SBV particles in PP buffer and incubated at 35°C; then immediately, and at 2 hour intervals thereafter, pupae in one of the SBV inoculated groups were challenged by inoculating each with 10 µl of a 10^{-2} diluted 'standard particle preparation' of KBV particles in PP buffer. Each challenged group was returned to the incubator until 72 hours after the initial

injection. Then, an extract was obtained from each injected pupa and tested for the presence of virus particles in serological gel diffusion tests as described in Section 4.2.4.1.

4.5.2.6 Results and Conclusions

The results, summarized in Table 4.5, indicate that all pupae in which SBV was given a 'start' of 10 hours or less were infected with KBV and none contained detectable concentrations of SBV particles. However, both viruses were isolated from some of the pupae in which SBV was given a 12-20 hour start; an increasing proportion yielded SBV and a decreasing proportion KBV. These results show that KBV suppresses the growth of SBV in pupae.

The ability of KBV to suppress SBV growth may have been because KBV grows faster than SBV or alternatively, there may be a more specific inhibitory phenomenon, such as competition for replication sites. These possibilities could be investigated in future studies.

Soon after I commenced my studies, when inapparent infections were first detected, a bee colony in the ACT was selected to monitor their monthly occurrence. This colony was monitored throughout my studies but the method of detecting the infections was modified after the results of the work on suppression were obtained. This study is reported in the following section.

TABLE 4.5 The number of pupae, infected with Kashmir bee virus (KBV) and sacbrood virus (SBV), detected when groups of 20 pupae were inoculated with SBV and later challenged at different times with KBV (*) (@).

TIME (HRS) THAT ELAPSED UNTIL CHALLENGED WITH KBV	# OF PUPAE INFECTED WITH: KBV	SBV (+)
0	20	0
2	20	0
4	20	0
6	20	0
8	20	0
10	20	0
12	20	5
14	20	10
16	20	20
18	10	20
20	1	19

(*): See text for experimental detail.

(@): 20 pupae were also inoculated with PP buffer. A pupa from this group yielded KBV.

(+): All extracts were also tested for antigens of other bee viruses, as described in the text, but none were detected.

4.6 OCCURRENCE OF INAPPARENT VIRUS INFECTIONS IN A BEE COLONY

The aim of this study was to determine the seasonal occurrence of inapparent virus infections in a normal bee colony.

4.6.1 Methods

Once a month (beginning in August 1981), 20 white-eyed pupae were removed from a single bee colony located in the ACT. Each pupa was injected with 10 µl of sterilized PP buffer and incubated at 35°C for 3 days. Then, an extract was obtained from each and tested for the presence of bee virus particles in gel diffusion tests as described in Section 4.2.4.1. In May 1983, after the conclusion of my studies on suppression of inapparent infections (Section 4.5), this colony was moved from Canberra, where brood is not produced during winter, to the Field Research Station of The Australian National University at Kioloa on the NSW South Coast, where brood is produced throughout the year. Starting then, and once a month thereafter, four groups of 20 white-eyed pupae were removed from the hive. Pupae in one group were each injected with 10 µl of sterilized PP buffer; individuals in another group were each injected with 10 µl of a 1:16 dilution of KBV antiserum in PP buffer; individuals in another group were each injected with 10 µl of a mixture of 1:16 dilutions of KBV and SBV

antisera in PP buffer, and individuals in the fourth group were each injected with 10 µl of a mixture of 1:16 dilutions of BQCV, KBV and SBV antisera in PP buffer. All injected individuals were incubated at 35°C for 3 days, then an extract was obtained from each and tested for the presence of virus particles by serological gel diffusion tests, as described above.

4.6.2 Results and Conclusions

Figure 4.2 shows the percentages of pupae with inapparent virus infections found each month (commencing in August 1981) in the groups of pupae which were injected with PP buffer, and Figure 4.3 shows the percentages of pupae with inapparent virus infections found each month (commencing in May 1983) in groups of pupae which were injected with either KBV antiserum in PP buffer, KBV and SBV antisera in PP buffer, BQCV, KBV and SBV antisera in PP buffer, or PP buffer.

The results in Figures 4.2 and 4.3 confirm my previous finding that an individual pupa may be inapparently infected with more than one virus, but when injected with PP buffer, only one virus is detected. For example, no extracts contained more than one virus but Figure 4.2 shows that during spring and early summer many pupae inoculated with PP buffer yielded KBV particles, and those few extracts that did not, mainly yielded SBV particles. However, Figure 4.3 shows that during spring and early

FIGURE 4.2 Percentages of pupae, from a normal colony, with monthly inapparent infections of cricket paralysis virus (+)(CrPV), Kashmir bee virus (●)(KBV), sacbrood virus (■)(SBV) and black queen cell virus (▲)(BQCV).

---: Dotted line indicates a sample interval of 2 months.

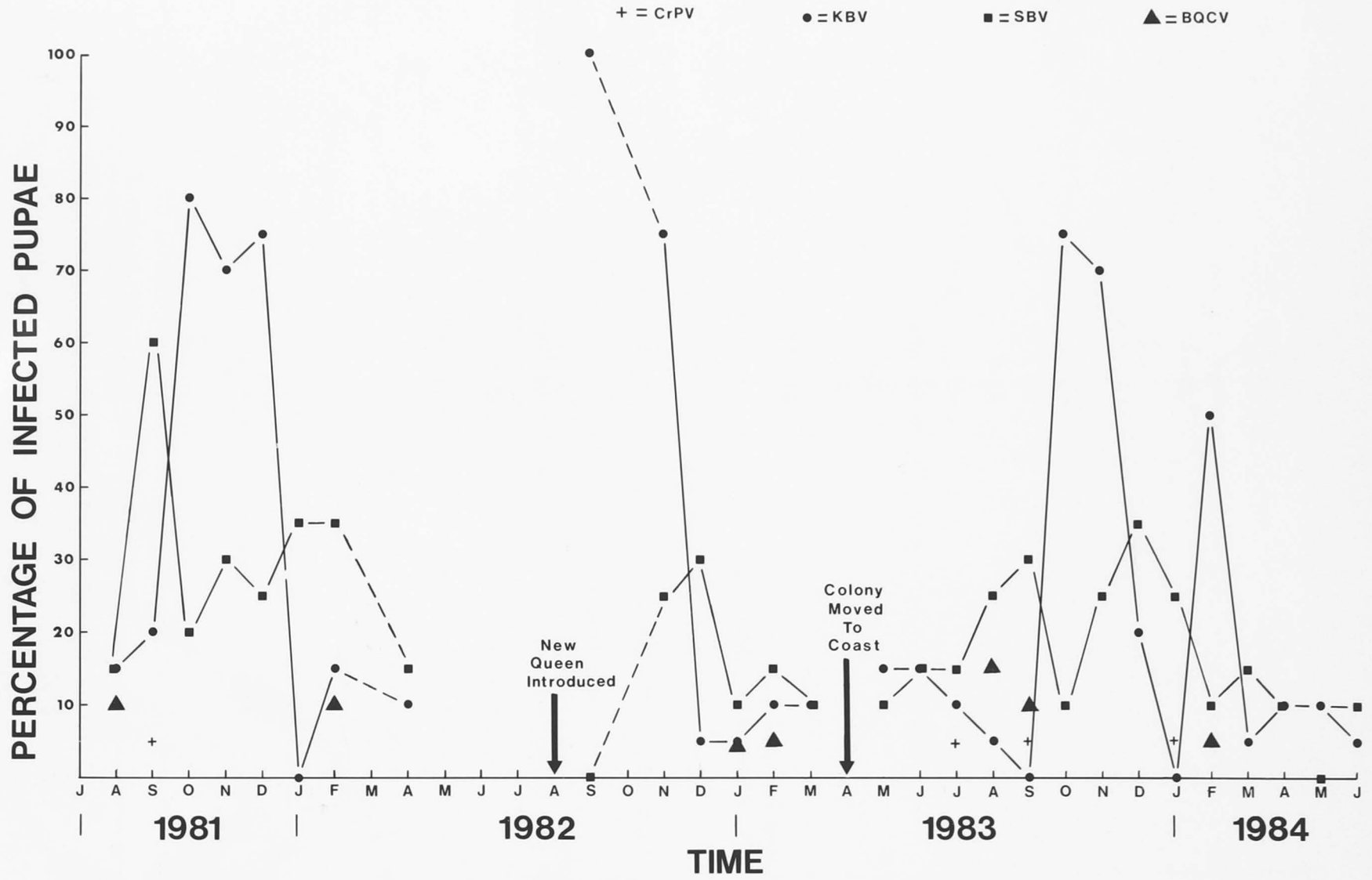
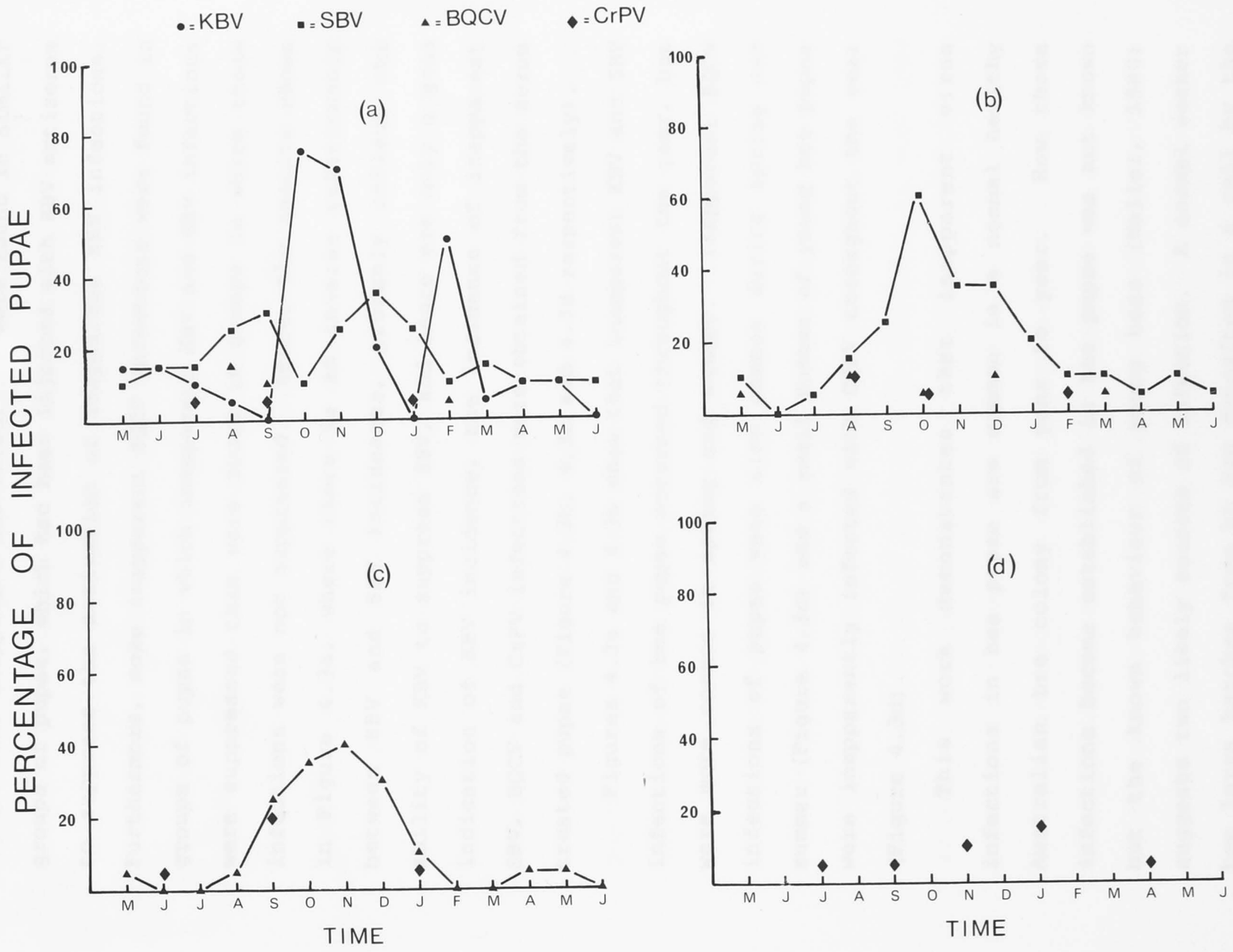

+ = CrPV
● = KBV
■ = SBV
▲ = BQCV
PERCENTAGE OF INFECTED PUPAE
100
90
80
70
60
50
40
30
20
10
New Queen Introduced
Colony Moved To Coast
J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J
1981
1982
1983
1984
TIME

FIGURE 4.3 The percentages of pupae (20 tested) from a normal colony with monthly inapparent infections of Kashmir bee virus (●)(KBV), sacbrood virus (■)(SBV), black queen cell virus (▲)(BQCV) or cricket paralysis virus (♦)(CrPV), after injection with 10mM potassium phosphate buffer (PP buffer) (a), a 1:16 dilution of KBV antiserum in PP buffer (b), 1:16 dilutions of both KBV and SBV antisera in PP buffer (c), or 1:16 dilutions of KBV, SBV and BQCV antisera in PP buffer (d).


● = KBV
■ = SBV
▲ = BQCV
◆ = CrPV
(a)
(b)
(c)
(d)
PERCENTAGE OF INFECTED PUPAE
TIME
TIME
100
80
60
40
20
M J J A S O N D J F M A M J

summer more inapparent SBV infections were found in similar groups of pupae, which had been injected with KBV antiserum to suppress the activation of inapparent KBV infections. Furthermore, more inapparent BQCV infections were found in groups of pupae in which inapparent KBV and SBV infections were suppressed than were found in groups in which those infections were not suppressed. Hence, the results shown in Figure 4.3a, where there is an inverse relationship between KBV and SBV incidences, probably reflect the ability of KBV to suppress SBV, and hence are only a good indication of KBV incidence; the incidence of inapparent SBV, BQCV and CrPV infections were obtained from the serum treated pupae (Figure 4.3b, 4.3c and 4.3d respectively).

Figures 4.3a and 4.3b show that inapparent KBV and SBV infections of bee pupae occurred throughout the year, but were more common in spring and summer. Inapparent BQCV infections of pupae were also common during spring and summer (Figure 4.3c) and a small number of young bee pupae were inapparently infected with CrPV throughout the year (Figure 4.3d).

This work demonstrates that inapparent virus infections in bee pupae are common in a normal healthy Australian bee colony from year to year. How these infections became established in bee pupae was not known but the known behaviour of young bees (Waller, 1980) suggests two likely sources of infection. A normal worker bee larva hatches from an egg deposited in a cell by the queen bee. The larva remains in its open cell for the next

five days, being fed by young nurse bees and moulting about once every 24 hours. For the first 24 hours of its life a larva is fed copious amounts of honey and 'royal jelly', a proteinaceous substance produced by the hypopharyngeal and mandibular glands of nurse bees. For the second 24 hours of its life a larva is fed the same type of food as the first 24 hours, but in smaller quantities. Thereafter, the larva is fed mainly honey and pollen. Just before its final larval moult and at the beginning of the prepupal stage, the larva is sealed in its cell with a wax covering by worker bees. The larva then pupates, metamorphoses, moults to become an adult insect, and finally gnaws its way out of the cell. A bee pupa, therefore, does not make physical contact with other bees and it is most likely that those pupae which become inapparently infected with virus are infected as eggs ~~in the egg stage~~ or, as larvae, by eating contaminated food.

It is not known whether bee viruses can be transmitted 'vertically' (i.e. from parent to offspring) and, furthermore, it would be extremely difficult to test this as bee viruses can only be propagated in bees which may already be inapparently infected with bee viruses. However, the possibility of pupae acquiring inapparent virus infections from contaminated food fed to larvae can be tested, and this study is reported in the following section.

4.7 ESTABLISHMENT OF INAPPARENT VIRUS INFECTIONS IN BEE PUPAE

In this study I tested whether larvae fed KBV or SBV contaminated food produced inapparently infected pupae. Larvae of different ages were first fed KBV or SBV particles or a 'control' extract, and those that developed into pupae were tested at the white-eyed stage for inapparent virus infections by injecting each with 10 µl of PP buffer, and incubating at 35°C for 3 days. Then, those larvae of an age shown to be most likely to develop into inapparently infected pupae were tested in other experiments to determine whether the concentration of virus particles fed to larvae was important for establishing inapparent KBV or SBV infections.

4.7.1 General Methods

4.7.1.1 Virus Preparations Fed to Larvae

An individual preparation containing SBV particles was obtained from a larva with sacbrood from a colony in the ACT by the methods described in Chapter 2. Similarly, a KBV particle preparation was obtained from a moribund pupa after an injection of 10 µl of PP buffer. To standardize the concentration of virus particles in each preparation, a 'standard particle preparation' (see Section 4.5.2.5) was made of each particle preparation used. These 'standard

particle preparations', prepared using PP buffer, were diluted in distilled water and for 'controls', PP buffer was similarly diluted in distilled water.

4.7.1.2 The Colonies

The bee colonies used in these studies were located at Kioloa, NSW.

4.7.1.3 Feeding Larvae

A comb containing larvae was removed from a colony and a 4 µl aliquot of a virus or 'control' extract was placed into the food surrounding each larva, using an Eppendorf pipette with sterile disposable tips. The comb was then returned immediately to the colony.

4.7.2 Testing for Larval Susceptibility

4.7.2.1 Determining the Age of Susceptible Larvae

4.7.2.1.1 Methods

An empty frame of wax comb was placed into each of two normal bee colonies on 29 May 1984, and 4 days later, most of the cells in both combs contained eggs. Three days later, most of the eggs hatched and, in the laboratory, all of the remaining unhatched eggs were removed. Then, each

side of the two combs was partitioned and marked to give 10 groups of one-day-old larvae on each frame. On a side of one comb (from colony 1), a group of one-day-old larvae was fed aliquots of a 10^{-2} diluted 'standard particle preparation' of SBV particles and the comb returned to the colony. On the following day a group of two-day-old larvae was similarly fed and on consecutive days thereafter groups representing three, four and five-day-old larvae were likewise fed. On the other side of this comb, comparable groups of larvae of different ages were similarly fed aliquots of a 10^{-2} dilution of PP buffer. The second comb (from colony 2) was treated in the same way except that a 10^{-2} diluted 'standard particle preparation' of KBV rather than SBV was used. On each occasion that the frames were treated, all newly laid eggs were removed.

After the final treatment, the combs were left in their colonies for 6 days, and in the laboratory, the individuals which had pupated in each group were carefully extracted from their cells, each injected with 10 µl of PP buffer, and incubated at 35°C for 3 days. An extract was obtained from each pupa and tested for the presence of bee virus particles in serological gel diffusion tests, as described in Section 4.2.4.1.

4.7.2.1.2 Results

Some larvae disappeared from their cells after SBV, KBV or PP buffer was added to their food. They were

probably ejected from the colony by nurse bees in the same way that larvae were removed from colonies when fed SBV particles by Bailey *et al.*, (1964), and when fed KBV preparations by Rhodes and Teakle (1978). Furthermore, some treated larvae remained in the prepupal stage at the conclusion of the experiment and could not be tested for inapparent infections.

Table 4.6 shows the number of larvae in each age group fed SBV or PP buffer (colony 1) or KBV or PP buffer (colony 2), together with the numbers and proportions (in brackets) of these larvae that disappeared, the numbers of remaining larvae which pupated and were injected with PP buffer, and the numbers (and proportions) of these pupae from colony 1 with inapparent SBV infections, and from colony 2 with inapparent KBV infections. As in previous experiments, only one virus was detected in individual extracts.

The results suggest that more pupae disappeared from the groups which were fed extracts containing virus particles than disappeared in groups fed PP buffer, and that greater proportions of larvae disappeared from groups fed undiluted virus preparations than from groups fed dilute virus preparations. In addition, more pupae appeared to become inapparently infected with SBV or with KBV when fed extracts of SBV or KBV respectively as larvae, than when fed PP buffer, and some groups appeared to contain higher proportions of inapparently infected pupae than others. These results was tested for statistical

TABLE 4.6 The number (and proportion) of pupae that became inapparently infected after feeding larvae with sacbrood virus (SBV) or KBV (KBV) particles(@)

COLONY #	TREATMENT(*)	AGE OF LARVAE WHEN TREATED (DAYS)	# OF LARVAE	# (AND PROPORTION) OF LARVAE THAT DISAPPEARED	# OF LARVAE THAT PUPATED(+)	# (AND PROPORTION) OF PUPAE INAPPARENTLY INFECTED WITH: SBV (COLONY 1)	KBV (COLONY 2)(^)
1	Fed SBV particles	1	110	46 (0.418)	60	29 (0.483)	
		2	83	36 (0.434)	13	8 (0.615)	
		3	53	20 (0.377)	7	4 (0.571)	
		4	43	14 (0.326)	4	2 (0.500)	
		5	91	14 (0.154)	60	15 (0.250)	
		TOTALS	380	130 (0.342)	144	58 (0.403)	
1	Fed PP buffer ('control')	1	54	11 (0.204)	36	5 (0.139)	
		2	63	12 (0.190)	41	6 (0.146)	
		3	95	10 (0.105)	82	6 (0.073)	
		4	39	2 (0.051)	27	2 (0.074)	
		5	91	1 (0.011)	64	7 (0.109)	
		TOTALS	342	36 (0.105)	250	26 (0.104)	
2	Fed KBV particles	1	104	65 (0.625)	37		6 (0.162)
		2	99	31 (0.313)	56		17 (0.304)
		3	55	35 (0.636)	8		1 (0.125)
		4	84	37 (0.440)	29		3 (0.103)
		5	20	4 (0.200)	8		0 (0.000)
		TOTALS	362	172 (0.475)	138		27 (0.196)
2	Fed PP buffer ('control')	1	56	12 (0.214)	40		1 (0.025)
		2	102	14 (0.137)	82		2 (0.024)
		3	91	13 (0.143)	76		1 (0.013)
		4	35	3 (0.086)	23		1 (0.043)
		5	44	0 (0.000)	34		2 (0.059)
		TOTALS	328	42 (0.128)	255		7 (0.074)

(*): See text for experimental details.
(+): Some larvae did not pupate.
(^): In colony 1, 17 pupae that were fed SBV as larvae and 15 fed PP buffer, became inapparently infected with KBV; in colony 2, 15 pupae fed KBV as larvae, and 19 fed PP buffer, became inapparently infected with KBV. No other viruses were detected.

(@): Larvae of different ages were fed with SBV, KBV or PP buffer (controls); the number of larvae that were removed from the colonies after treatment were recorded and surviving larvae that pupated were tested for inapparent virus infections as described in the text.

significance using the GENSTAT package described in Section 4.2.2.

4.7.2.1.3 Larvae That Disappeared

Tests were done to determine whether more larvae disappeared from the groups fed KBV or SBV ('test' groups) than from groups fed PP buffer ('control' groups).

In colony 1 the proportion of total number of larvae that disappeared from the control group is 36/342 = 0.105, so the proportion remaining is 306/342 = 0.895. The 'odds' of the proportion that disappeared to those that remained are then 0.105:0.895, or 0.118:1. For total number of larvae that disappeared from the test group, the odds are 0.520:1. The odds-ratio of the test group relative to the control group is thus 0.520/0.118 = 4.4. Fitting a logistic regression model to the data gave an odds-ratio of 4.32 with approximate 95% confidence limits of (2.8, 6.6). Since these limits do not include unity, it can be concluded that a significantly greater proportion of larvae disappeared when fed SBV than when fed PP buffer.

Likewise, in colony 2, a significantly greater proportion of larvae disappeared when fed KBV than when fed PP buffer. The fitted odds-ratio of test to control group was 6.17 with approximate 95% confidence limits of (4.11, 9.28).

Tests were also done to determine whether there were significant differences between the proportions of larvae

that disappeared when fed either SBV or KBV as 1, 2, 3, 4 or 5-day-old larvae. These ratios were fitted relative to 2-day-old larvae because they were used in a subsequent experiment to determine whether the concentration of virus particles fed to larvae determined the proportion of pupae that become inapparently infected (Section 4.7.2.2). The fitted odds-ratios of larvae that disappeared each day relative to 2-day-old fed larvae, together with the approximate 95% confidence limits are shown in Table 4.7. These results show that significantly higher proportions of 1, 2, and 3-day-old than 5-day-old larvae were removed when fed SBV or KBV.

4.7.2.1.4 Inapparently Infected Pupae

Tests showed that in colony 1, a significantly greater proportion of pupae became inapparently infected with SBV in the test group than in the control group. The fitted odds-ratio of test to control group was 5.91 with approximate 95% confidence limits of (3.22, 10.85). Similarly, a significantly greater proportion of pupae from colony 2 became inapparently infected with KBV in the test group than in the control group. The fitted odds-ratio of test to control group was 8.62 with approximate 95% confidence limits of (3.58, 20.61).

Other tests showed there were no significant differences in the proportions of pupae that became infected with KBV when fed KBV as 1, 2, 3, 4 or 5-day-old

TABLE 4.7 The fitted odds-ratios and approximate confidence limits of larvae which disappeared from colonies when fed sacbrood virus (SBV) or Kashmir bee virus (KBV) at different ages, relative to 2-day-old SBV or KBV fed larvae (+).

COLONY #	TREATMENT	AGE OF LARVAE (DAYS)	ODDS-RATIO	95% CONFIDENCE LIMITS: LOWER	UPPER
1	Fed SBV	1	0.95	0.58	1.56
"	" "	3	0.67	0.38	1.18
"	" "	4	0.50	0.25	0.97
"	" "	5	0.18	0.09	0.35
2	Fed KBV	1	2.89	1.78	4.71
"	" "	3	2.34	1.39	3.96
"	" "	4	1.31	0.77	2.23
"	" "	5	0.29	0.10	0.86

(+): See text for experimental details.

larvae, however, there were significant differences in the proportions of pupae that became infected with SBV at different ages. The fitted odds-ratios of pupae that became infected with SBV when fed SBV as larvae, relative to 2-day-old SBV fed larvae, together with the approximate 95% confidence limits are shown in Table 4.8. These results show that higher proportions of inapparent SBV infections were detected in 1, 2, 3 and 4-day-old SBV fed larvae than in 5-day-old SBV fed larvae.

4.7.2.1.5 Discussion

The results from this experiment clearly show that larvae which eat KBV or SBV contaminated food develop into KBV or SBV inapparently infected pupae. Higher proportions of inapparent SBV infections were found in pupae that were fed SBV particles when they were 1, 2, 3 and 4-day-old larvae than when fed as 5-day-old larvae, suggesting that younger larvae may be more susceptible for developing inapparent SBV infections than older larvae. However, more young larvae than old larvae disappeared from the colony after they were fed SBV particles.

Two-day-old larave were used in the following experiment to test whether the concentration of KBV and SBV particles fed to larvae determined the proportion of pupae that become inapparently infected. The statistical tests showed that larvae of this age were as susceptible to KBV and SBV as 1 and 3-day-old larvae.

TABLE 4.8 The fitted odds-ratios and approximate confidence limits of pupae which became infected with sacbrood virus (SBV) when fed SBV at different ages, relative to 2-day-old SBV fed larvae (*).

COLONY #	LARVAL TREATMENT	ODDS-RATIO	95% CONFIDENCE LIMITS: LOWER	UPPER
1	Fed SBV on day 1	0.78	0.34	1.80
"	" " " " 3	0.49	0.19	1.26
"	" " " " 4	0.50	0.14	1.80
"	" " " " 5	0.35	0.15	0.82

*: See text for experimental details.

4.7.2.2 The Effects of Virus Particle Concentration on the Establishment of Inapparent Virus Infections in Bee Pupae

4.7.2.2.1 Methods

Two frames of wax comb containing cells of one-day-old larvae were obtainined from each of two normal bee colonies on 28 June 1984 by the method described in Section 4.7.2.1. All unhatched eggs were removed from cells of the four combs using sterilized forceps, then the frames were returned immediately to the parent colony. The following day the four frames of comb were removed to the laboratory and both sides of each comb partitioned to give 7 groups of 2-day-old larvae per frame of comb. Individual larvae in 7 groups in 2 combs from the same colony were fed aliquots of either undiluted PP buffer or a 10^{o}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or a 10^{-6} diluted 'standard particle preparation' of SBV particles. Undiluted buffer or similarly diluted aliquots of a 'standard particle preparation' of KBV particles were fed to individual larvae in 7 groups in the other 2 combs from the second colony.

Immediately after the last group of larvae was treated, the 4 combs were returned to their parent colonies where they remained until 4 days after the larvae were capped. Then, all pupae were carefully removed, injected with 10 µl of PP buffer, and incubated at 35^{o}C for 3 days,

after which time an extract was obtained from each pupa and tested for bee virus particles in gel diffusion tests, as described in Section 4.2.4.1.

4.7.2.2.2 Results

Table 4.9 shows the number of two-day-old larvae in each treated group, the number and proportion (in brackets) of treated individuals that disappeared, the number of surviving pupae in each treated group and the number (and proportion) of surviving pupae with inapparent SBV infections (colony 1) or inapparent KBV infections (colony 2). As found in previous experiments, there was no evidence of mixed infections in individual extracts.

The results shown in Table 4.9 suggest that greater proportions of larvae disappeared from groups fed undiluted (10^{0}) SBV or KBV particle preparations than from groups fed diluted preparations, and that some groups fed SBV or KBV particles yielded greater proportions of SBV and KBV inapparently infected pupae respectively than others.

These results were tested for significance using logistic regression models as described in Section 4.7.2.1.3, except that the odds-ratios were fitted relative to the respective group of larvae fed undiluted PP buffer ('control' group). The fitted odds-ratios of larvae that disappeared from the replica groups of larvae fed SBV or KBV particles ('test' groups), relative to their respective control groups, together with the approximate 95%

TABLE 4.9 The number and proportion of pupae that became inapparently infected after feeding 2-day-old larvae with dilutions of sacbrood virus (SBV) or Kashmir bee virus (KBV) particles.(@)

COLONY No.: COMB No.	TREATMENT(*)	No. OF LARVAE TREATED	No.(AND PROPRTION) OF LARVAE THAT DISAPPEARED	No. OF LARVAE THAT PUPATED(+)	No.(AND PROPORTION) OF PUPAE INAPPARENTLY INFECTED WITH: SBV(COLONY 1)	KBV(COLONY 2)(#)
	Fed 10^{0} SBV	82	67 (0.817)	6	0 (0.00)	
	" 10^{-1} "	56	29 (0.518)	15	0 (0.00)	
1:1	" 10^{-2} "	86	26 (0.302)	44	5 (0.114)	
	" 10^{-3} "	94	1 (0.011)	73	17 (0.233)	
	" 10^{-4} "	65	2 (0.031	55	15 (0.273)	
	" 10^{-6} "	43	1 (0.023)	42	12 (0.286)	
	TOTALS	426	126 (0.296)	235	49 (0.208)	
	Fed 'control' extract	52	3 (0.058)	32	3 (0.094)	
	Fed 10^{0} SBV	77	73 (0.948)	0	0 (0.00)	
	" 10^{-1} "	134	72 (0.537)	27	9 (0.333)	
1:2	" 10^{-2} "	110	12 (0.109)	62	20 (0.322)	
(Replica	" 10^{-3} "	127	4 (0.031)	101	11 (0.109)	
of 1:1)	" 10^{-4} "	90	3 (0.033)	71	10 (0.141)	
	" 10^{-6} "	134	2 (0.015)	96	11 (0.114)	
	TOTALS	672	166 (0.247)	268	61 (0.228)	
	Fed 'control' extract	82	5 (0.061)	61	3 (0.049)	
	Fed 10^{0} KBV	141	115 (0.816)	3		2 (0.667)
	" 10^{-1} "	101	11 (0.109)	35		10 (0.286)
2:1	" 10^{-2} "	131	23 (0.175)	66		10 (0.152)
	" 10^{-3} "	117	22 (0.188)	65		15 (0.231)
	" 10^{-4} "	128	14 (0.109)	105		7 (0.067)
	" 10^{-6} "	111	16 (0.144)	84		8 (0.095)
	TOTALS	729	201 (0.276)	358		52 (0.145)
	Fed 'control' extract	55	3 (0.054)	42		2 (0.048)
	Fed 10^{0} KBV	101	94 (0.931)	3		1 (0.333)
	" 10^{-1} "	97	39 (0.402)	41		15 (0.366)
2:2	" 10^{-2} "	126	12 (0.095)	95		6 (0.063)
(Replica	" 10^{-3} "	180	11 (0.061)	147		14 (0.095)
of 2:1)	" 10^{-4} "	119	18 (0.151)	92		20 (0.217)
	" 10^{-6} "	97	14 (0.144	66		6 (0.091)
	TOTALS	720	188 (0.261)	444		62 (0.140)
	Fed 'control' extract	64	5 (0.078)	39		1 (0.026)

(*): For details of treatments, see text.
(+): Some larvae did not pupate
(#): 12 pupae from colony 1 that were fed dilutions of SBV particles, and 3 fed PP buffer, became inapparently infected with KBV; 17 pupae from colony 2 that were fed dilutions of KBV particles, and 2 fed PP buffer, became inapparently infected with SBV. No other viruses were detected.

(@): The number of larvae that disappeared from the colonies after treatment were recorded and surviving larvae that pupated were tested for inapparent virus infections as described in the text.

confidence limits are shown in Table 4.10. These results show that a significantly greater proportion of larvae disappeared when fed 10^{o} SBV or KBV particles than when fed diluted SBV or KBV particle preparations or PP buffer.

The fitted odds-ratios of pupae which became inapparently infected with KBV or SBV in the replica test groups, relative to their respective control groups, together with the approximate 95% confidence intervals are shown in Table 4.11. These results show that groups fed 10^{o}, 10^{-1}, and 10^{-2} dilutions of SBV or KBV particles produced significantly greater proportions of SBV or KBV inapparently infected pupae respectively than did larvae fed PP buffer, however, the differences in the proportions of SBV or KBV inapparently infected pupae in groups fed SBV or KBV particles respectively (Table 4.9) were not statistically significant.

4.7.2.2.3 Discussion

The differences between the results of replica experiments shown in Table 4.9 indicate that not all variables were accounted for, and controlled by, the experimental design used. For example, an important variable which could not be controlled was the genetic susceptibility of larvae to SBV or KBV. In addition, the experiments were done during autumn and winter when there are very large changes in weather and availability of food. However, despite these problems the results showed that

TABLE 4.10 The fitted odds-ratios and approximate confidence limits of larvae which disappeared from colonies when fed dilutions of sacbrood virus (SBV) or Kashmir bee virus (KBV) as 2-day-old larvae, relative to 2-day-old larvae fed PP buffer (controls).

LARVAL TREATMENT(*)	ODDS-RATIO	95% CONFIDENCE LIMITS: LOWER	UPPER
Fed 10^{0} SBV	116.05	27.32	492.99
" 10^{-1} "	17.87	4.90	65.14
" 10^{-2} "	3.79	0.99	14.51
" 10^{-3} "	0.36	0.05	2.47
" 10^{-4} "	0.53	0.05	2.49
" 10^{-6} "	0.27	0.03	2.65
Fed 10^{0} KBV	88.23	11.05	704.58
" 10^{-1} "	4.66	0.61	35.82
" 10^{-2} "	2.18	0.27	17.42
" 10^{-3} "	1.73	0.22	13.84
" 10^{-4} "	2.08	0.25	16.90
" 10^{-6} "	2.34	0.29	19.05

(*): See text for experimental details.

TABLE 4.11 The fitted odds-ratios and approximate confidence limits of pupae which became inapparently infected with sacbrood virus (SBV) or Kashmir bee virus (KBV) when fed dilutions of SBV or KBV respectively as 2-day-old larvae, relative to 2-day-old larvae fed PP buffer (controls).

LARVAL TREATMENT(+)	ODDS-RATIO	95% CONFIDENCE LIMITS: LOWER	UPPER
Fed 10° SBV	0.01	*	*
" 10-1 "	3.94	0.33	47.43
" 10-2 "	4.48	0.54	37.22
" 10-3 "	2.77	0.35	22.14
" 10-4 "	3.60	0.43	29.87
" 10-6 "	2.89	0.35	23.97
Fed 10° KBV	26.05	0.86	788.71
" 10-1 "	12.81	1.48	110.61
" 10-2 "	2.86	0.32	25.67
" 10-3 "	4.14	0.50	34.36
" 10-4 "	4.14	0.50	34.36
" 10-6 "	2.66	0.29	24.41

(+): See text for experimental details.

* : No pupae infected

pupae become inapparently infected with SBV or KBV when they eat SBV or KBV contaminated food as larvae. This was also shown in the previous experiment. In addition, the results showed that most larvae which were fed undiluted preparations of each virus disappeared from the colony before they pupated. This suggests that perhaps the KBV preparations, which Rhodes and Teakle (1978) fed to young larvae when they unsuccessfully attempted to demonstrate 'Kashmir bee virus disease' (Section 3.4), contained high concentrations of KBV particles.

4.8 SUMMARY AND DISCUSSION OF RESULTS OF STUDIES OF INAPPARENT VIRUS INFECTIONS IN BEE PUPAE

The following points briefly summarize my major findings of studies on inapparent infections.

(1) Apparently healthy young bee pupae in normal bee colonies were shown to be inapparently infected with BQCV, CrPV, KBV and SBV. These infections were not detected in individual pupae taken directly from a colony, but were detected after the viruses had been activated. To activate the viruses, pupae were injected with PP buffer (or other salt solutions), and held at 35°C for 3 days. However, some inapparent SBV infections were activated merely by keeping uninjected pupae at 35°C for 3 days.

(2) Adult bees, produced from pupae with inapparent KBV and SBV infections, were themselves inapparently infected,

and such infections were activated by injections of PP buffer.

(3) Inapparent BQCV, KBV and SBV infections in bee pupae were suppressed by injecting infected pupae with 1:16 dilutions of specific antisera. However, more SBV infections were found in groups of pupae in which inapparent KBV infections were suppressed than were found in groups in which those infections were not suppressed. In addition, more BQCV infections were found in groups of pupae in which SBV and KBV infections were suppressed than were found in groups in which those infections were not suppressed. These results showed that some pupae, previously thought to be inapparently infected with only one virus, were probably infected with several viruses, but when activated, only one virus grew to detectable concentrations. These, and other results from experiments in which pupae were injected with SBV and later challenged with KBV, suggested that KBV suppresses the growth of SBV.

(4) Inapparent BQCV, KBV and SBV infections were found in bee pupae in a normal Australian bee colony throughout the year, but were more common in spring and summer. In the same colony, a small number of pupae were found to be inapparently infected with CrPV throughout the year. This virus has not been previously reported in bee colonies in nature.

(5) Bee pupae became inapparently infected with SBV and KBV when, as larvae, they were fed extracts containing particles of each virus. In addition, many larvae

disappeared from colonies when fed undiluted preparations of each virus, and 1, 2, 3 and 4-day-old larvae were significantly more susceptible than 5-day-old larvae for establishing inapparent SBV infections.

Thus my work demonstrated that larvae become inapparently infected with KBV or SBV when they eat contaminated food and that they carry these infections into the pupal and adult bee stages. However, even though pupae were shown to acquire inapparent KBV and SBV infections as larvae, transovarial transmission was not tested and thus cannot be excluded as a possible source of infection.

The mechanisms by which phosphate buffer activated inapparent KBV infections during my studies are not known. Perhaps the injection of phosphate buffer into inapparently infected pupae damaged the virus containment mechanism in some way. The significance of inapparent KBV and SBV infections in the life cycle of each virus will be discussed in the general discussion that follows Chapter 5.

My finding are in general agreement with those of Dall (1985) who reported that inapparent infections of KBV and SBV in bee pupae are common in Australian colonies, and that inapparent SBV infections may be found in uninjected pupae. Dall also found no evidence of mixed inapparent infections in individual pupae. However, an interesting finding of my work was that a bee pupa may be inapparently infected with several viruses and that activation causes the particles of only one to grow to detectable concentrations. Furthermore, the combination of viruses

inapparently infecting a pupa determines which virus grows when activated (see (3) above). The underlying mechanisms determining which virus will grow is not known.

By using specific antisera to suppress inapparent virus infections in bee pupae, a method was found for propagating bee viruses without contamination by serologically distinct viruses, thereby allowing characterization studies using virus particles of each serotype. These studies are described in the following chapter.

CHAPTER 5

STRAIN VARIATION STUDIES

5.1 INTRODUCTION

Virus particles which have successfully infected susceptible cells use the cellular synthetic mechanisms to replicate. However, progeny viral genomes may differ in sequence from those of the parent as a result of spontaneous mutations, nucleotide substitutions or deletions, or replicase errors during transcription.

Organisms that have DNA as their genetic material are thought to have error-suppressing and proof-reading mechanisms associated with replication and these decrease the error rate during genome replication. However, similar mechanisms have not been reported in association with enzymes that replicate RNA genomes. Reanney (1982) suggested that the absence of error-suppressing and repair mechanisms may explain why RNA viral genomes have a greater mutation rate than DNA genomes.

Domingo *et al.*, (1978) investigated the differences between isolates from a culture of Qβ phage, a virus with a single-stranded RNA genome of about 3 Kb. They found that, on average, every genome in a population had one different nucleotide from the modal sequence of the population. However, when any recognisably different variant genome was

mixed with the modal population and propagated, the variant disappeared after subculturing 10-20 times. They concluded that viruses with RNA genomes probably produce mutants more quickly than DNA viruses, but these are strongly selected against and are quickly lost, and in this way, the modal population persists. However, the viral population may change when the selective pressures change as, for example, when the host itself changes, either by natural evolution or as a result of 'artificial' selection by man. Such changes are very noticeable when the conditions are radically changed as, for example, when viruses are cultured in persistently infected cell cultures.

I use the term 'variant' to describe any novel virus isolate, but describe naturally occurring variants as strains (Gibbs and Harrison, 1976). The study of strains and variants of insect viruses is of great importance for virologists because they may complicate the process of virus identification. Furthermore, the special characteristics of variants may be used as labels in experiments to reveal aspects of the ecology of a virus.

Honey bees have been maintained by man in Europe and the United Kingdom for at least three centuries and have only been recently cultivated in other parts of the world. Many bee viruses, isolated in countries where honey bees have been recently established, are strains that differ from those common in north-west Europe and thus may reflect radiative adaptation of the viruses in a new environment or the aquistion, by honey bees, of viruses from other insects

in their new environment. For example, Kashmir bee virus (KBV) was first isolated from the south-east Asian hive bee, *Apis cerana*, and has not been reported from honey bees in the northern hemisphere. However, Bailey *et al.*, (1979) isolated three serologically distinct strains of KBV in Australian honey bees. This led Bailey (1981) to suggest that KBV found in Australian honey bees has recently been acquired from a native insect species.

A study of strain variation in bee viruses in Australia seemed warranted to determine the frequency and distribution of the strains of KBV reported by Bailey, *et al.*, (1979). Furthermore, it seemed worthwhile to determine whether there were comparably different strains of sacbrood virus (SBV), as this could possibly explain the widespread outbreak of SBV in honey bee colonies in eastern Australia in 1981 and 1982 (Chapter 3), and the geographical distribution of those strains might give some clues about the cause of the outbreak.

In this chapter I first report studies to determine the serological relatedness of SBV particle isolates, together with a comparison of their electrophoretic mobilities, isoelectric points and sizes of their coat proteins, and also describe similar studies using KBV isolates. Then, I describe some preliminary results from studies using restriction endonuclease enzymes to digest complementary DNA (cDNA)/RNA hybrid duplexes of KBV isolate genomes.

5.2 SAMPLE PREPARATION

The virus isolates were propagated in white-eyed honey bee pupae as described in Section 2.2, except that the SBV isolates were propagated after mixing inocula of them with 1:16 dilutions of KBV and cricket paralysis virus (CrPV) antisera before injection, in order to suppress possible contamination with those viruses.

KBV and SBV particles, in individual and group preparations, were obtained by the methods described in Section 2.3. Group preparations of both viruses were further purified in sucrose gradients as also described in Section 2.3. Electron micrographs of typical group preparations of SBV and KBV particles, purified in this way, are shown in Figures 5.1 and 5.2 respectively.

5.3 STRAIN VARIATION STUDIES USING SBV ISOLATES

Various methods were used to compare isolates from different colonies in eastern Australia.

5.3.1 Gel Diffusion Tests

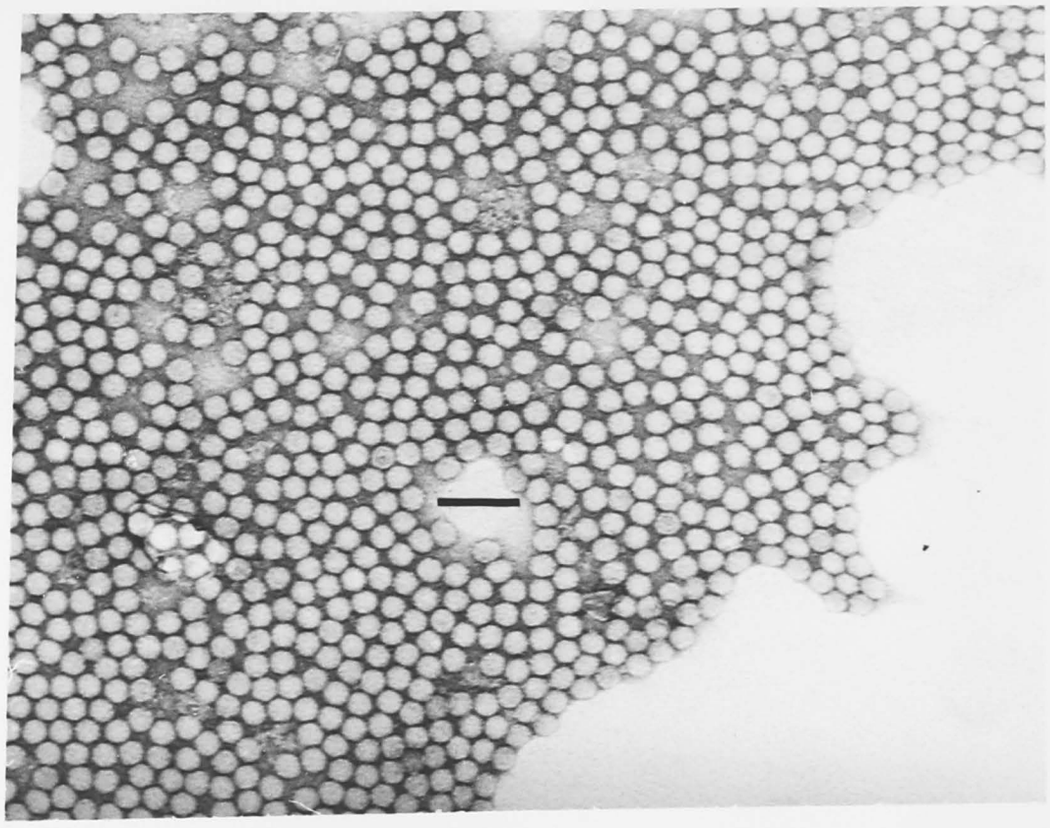
Individual preparations of SBV were obtained from larvae collected from each of the 82 honey bee colonies sampled during the surveys described in Section 3.2.1. They were tested for the presence of virus particles by the gel diffusion method (using the antisera listed in Section

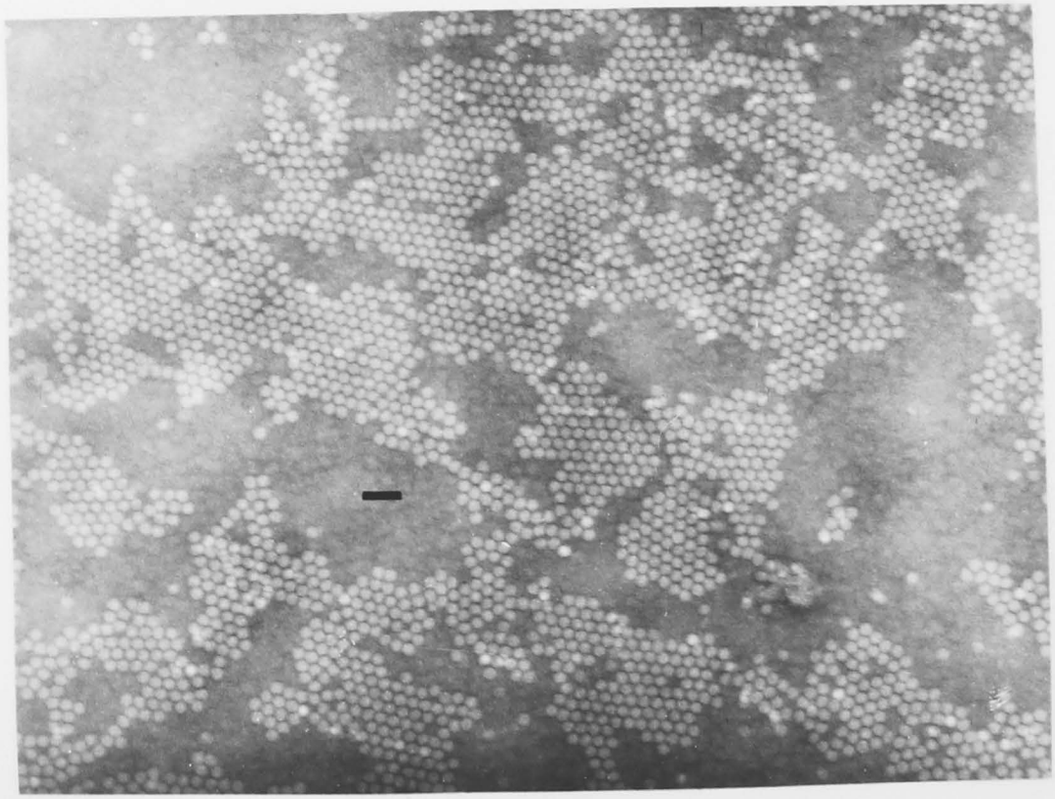
FIGURE 5.1 Micrograph of negatively stained sacbrood virus particles.

Bar represents 100 nm.

FIGURE 5.2 Micrograph of negatively stained Kashmir bee virus particles.

Bar represents 100 nm.





2.4, except that my BQCV, CBPV, KBV, and SBV antisera were substituted for Dr Bailey's) and by examining negatively stained preparations by electron microscopy; only SBV particles were detected.

The serological relationships of the isolates were assessed in gel diffusion tests by determining whether the precipitin band, produced by the particles of an isolate with homologous antiserum, fused, spurred or did not react with the precipitin bands produced by other SBV isolates placed in neighbouring wells in the gel.

5.3.1.1 Results

All 82 SBV isolates tested were serologically indistinguishable in gel diffusion tests. The precipitin lines produced by the particles of each isolate completely fused with those of other isolates in all replicate tests of all pairwise comparisons. One such test result is shown in Figure 5.3.

5.3.2 Electrophoretic Mobilities of SBV Particle Isolates

Electrophoretic mobility is a measure of the rate of migration of charged macromolecules, such as virus particles, in an electric field and has been a useful method for characterizing and distinguishing between strains of viruses and other macromolecules. The mobility of a virus particle in an electric current depends on its

nett charge, which is determined by the number and type of charged amino acids on its exposed surface. Therefore, virus particles with different amino acid sequences in their coat proteins might be differentiated by electrophoresis.

The SBV particle extracts used in the experiments described in Section 5.3.1, together with SBV particles in individual preparations obtained from infected larvae from colonies at Moruya and Richmond in New South Wales (NSW), Millicent and Kingston in South Australia (SA), and from Canberra in the Australian Capital Territory (ACT), were compared by electrophoresis. Also compared were SBV particles in group preparations obtained from larvae with sacbrood, collected from Leyburn, Kuranda, Herberton and Rockhampton in Queensland, Millicent and Kingston in SA, Tamworth, Moruya, Grafton, Richmond and Coonabarabran in NSW, and from Canberra.

Horizontal slab electrophoresis was done as described in Section 2.5.3. Virus particles in the gels were located by examining the gel on a fluorescent background for ultra-violet (U.V.) absorbing spots, or staining each gel in 0.25% (w/v) Coomassie Brilliant Blue (CBB), or alternatively, by immunoelectrophoresis as also described in Section 2.5.3. The Rø value of each particle isolate was measured (Section 2.5.3) and compared with the Rø values of each of the other isolates.

The CBB staining method was not used for locating particles in gels in which partially purified individual

preparations had been tested, as these preparations contained contaminating proteins of the host; they had a range of electrophoretic mobilities, stained strongly and obscured the virus 'spots'. Therefore, virus particles in these gels were detected serologically or by U.V. absorption. Group preparations, which were purified by sucrose gradient centrifugation, contained less contaminating protein, and the virus particles in them gave clear oval 'spots' that were easily detected by CBB staining.

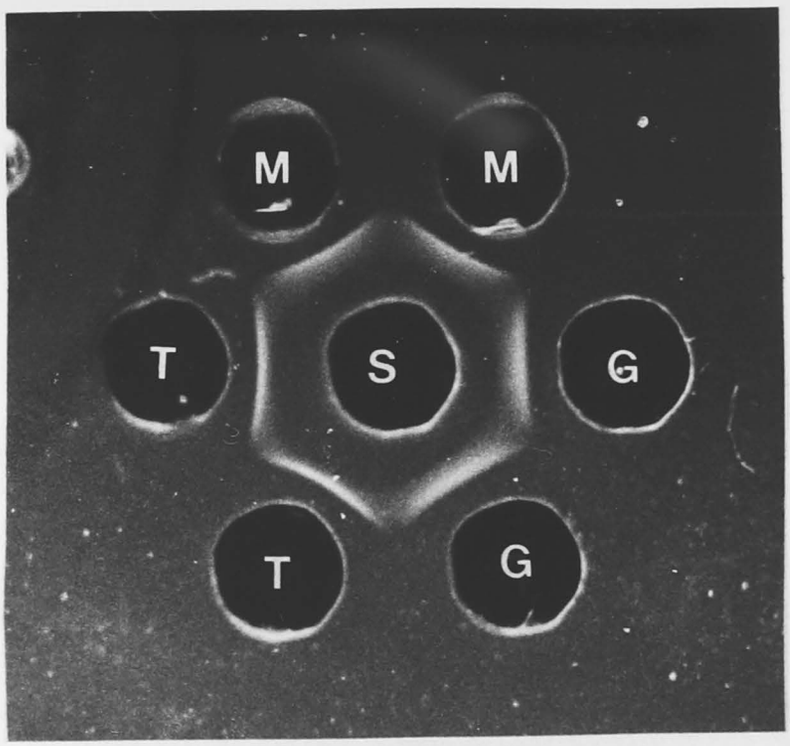
5.3.2.1 Results and Conclusions

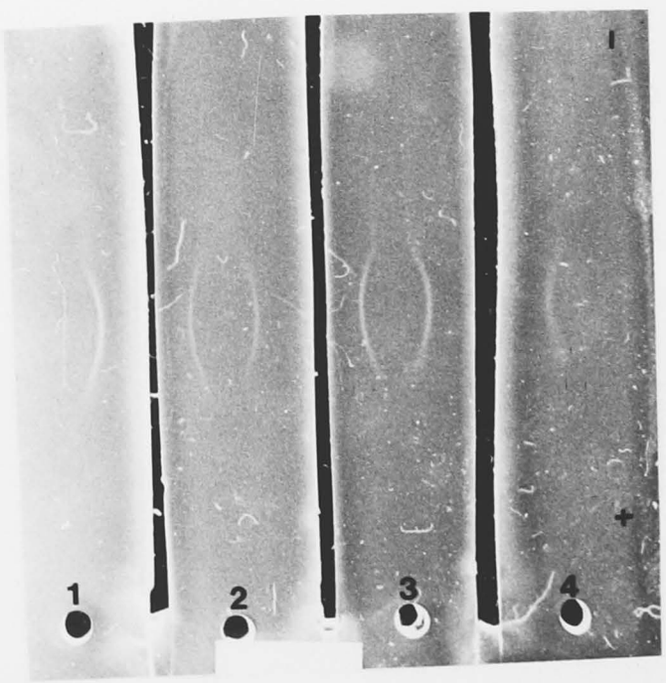
Figure 5.4 shows the results of a typical immuno-electrophoresis test comparing the mobilities of the particles of four SBV isolates.

All the SBV particle isolates in individual preparations, and 8 of the 11 SBV particle isolates in group preparations, had the same electrophoretic mobilities; each gave an Rø value of 0.22. The mobilities of the particles of the remaining three SBV isolates in group preparations obtained from larvae from colonies at Herberton, Richmond and Canberra, had Rø values of 0.51. However, SBV particles in individual preparations obtained from larvae collected from these same three colonies gave electrophoretic Rø values of 0.22, suggesting that the faster migrating particles had mobilities which were altered, perhaps during purification or subsequent storage.

FIGURE 5.3 Precipitin line formation produced in a gel diffusion test using sacbrood virus antiserum (S), and sacbrood virus particle isolates from Mareeba (M), Gympie (G), and Tamworth (T).

FIGURE 5.4 Precipitin line formation in an immunoelectrophoresis test using sacbrood virus antiserum and sacbrood virus particle isolates from Leyburn (1), Warwick (2), Mackay (3) and Kemsey (4).


M
M
T
S
G
T
G


1
2
3
4
+

The following experiment was done to test these possibilities.

5.3.2.2 Experiment 1

A fresh individual and group preparation of the Canberra isolate of SBV was obtained and immediately after purification, each preparation was divided into three aliquots; one was frozen at -20°C, one held at 4°C and the remaining aliquot was kept at room temperature. Then, every 4 days, the serological relatedness and the electrophoretic mobility of the SBV particles in each aliquot was examined.

5.3.2.3 Results and Conclusions of Experiment 1

The Rø values are shown in Table 5.1. The Rø's of the particles in both preparations were the same immediately after purification but became greater as both preparations aged. The change in mobility occurred sooner in group preparations in which the particles had been purified in sucrose gradients and kept at room temperature or at 4°C than in similarly purified preparations kept frozen at -20°C or for particles in partially purified individual preparations. The mobility of particles in the individual preparations changed sooner when kept at room temperature than at 4°C, but did not change their mobility when stored at -20°C (Table 5.1). However, particles which had changed

TABLE 5.1 Electrophetic mobilities, expressed as Rø values, of sacbrood virus (SBV) particles in individual and group preparations stored at -20°C, 4°C and room temperature (*).

AGE OF ISOLATE (DAYS)	INDIVIDUAL PREPARATIONS STORED AT:			GROUP PREPARATIONS STORED AT:		
	-20°C	4°C	Room Temp.	-20°C	4°C	Room Temp.
0	.22	.22	.22	.22	.22	.22
4	.22	.22	.22	.22	.51	.51
8	.22	.22	.22	.22	.51	.51
12	.22	.22	.22	.22	.51	.51
16	.22	.22	.51	.51	.51	.51
20	.22	.22	.51	.51	.51	.51
24	.22	.36	.51	.51	.51	.51
28	.22	.36	.51	.51	.51	ND+
32	.22	.51	.51	.51	.51	ND
36	.22	.51	.51	.51	ND	ND

(*): See text for experimental details.
+: ND = not determined.

their electrophoretic mobility remained serologically indistinguishable in gel diffusion tests from particles which had not changed mobility.

The three electromorphs of SBV in this experiment (Table 5.1) had Rø values similar to those of SBV which I reported in an earlier study (Anderson, 1980). However, as group preparations were used in that earlier study, it is likely the three electromorphs had changed their mobility during, or after, purification, and were not different strains as had been concluded.

Other viruses have been reported to change their electrophoretic mobilities after purification. For example, Niblett and Semancik (1969) observed two components with different electrophoretic mobilities in purified preparations of cowpea mosaic and bean pod mottle virus particles. The faster components were particles that had lost small peptides from their coat proteins as a result of hydrolysis by host proteases. Although it is not known what caused the SBV particles to change their mobilities, it is possible that contaminating enzymes caused proteolytic changes.

5.3.3 Isoelectric Points of SBV Particle Isolates

The isoelectric point is the pH value of the suspending medium at which a macromolecule, such as a virus particle, has no nett charge imbalance, and thus will not migrate in an electric field. At all other pH values the

particles will have a nett positive or negative charge and thus will migrate. The isoelectric focussing technique is used to measure the isoelectric point and, as the method is relatively fast, simple and widely used to distinguish between related proteins, it seemed worthwhile to assess whether it could distinguish between isolates of SBV particles.

Group preparations were obtained from larvae with sacbrood collected from colonies located at Canberra, Coonabarabran, Grafton, Herberton, Kuranda, Millicent, Moruya, Richmond and Tamworth. Each extract was tested for the presence of virus particles by electron microscopy and by gel diffusion serological tests, using the 10 different antisera listed in Section 2.4; only SBV particles were found in each.

The isoelectric points of the particles of each isolate were estimated by the methods described in Section 2.5.4.

5.3.3.1 Results

The isoelectric points of the particles in all the SBV preparations were indistinguishable and were approximately pH 5.0. Because isoelectric focussing did not reveal any differences, I decided to test SBV isolates for differences in the molecular weights of their coat proteins.

5.3.4 Molecular Weights of Coat Proteins of SBV Particle Isolates

The SBV isolates in group preparations which were tested in the previous experiment (Section 5.3.3), except the Moruya isolate, together with 2 group preparations of a Canberra isolate of SBV which had particles with electrophoretic RØ values of either 0.22 or 0.51, were used in these experiments.

The molecular weights of the capsid proteins of each isolate were estimated by the method described in Section 2.6.

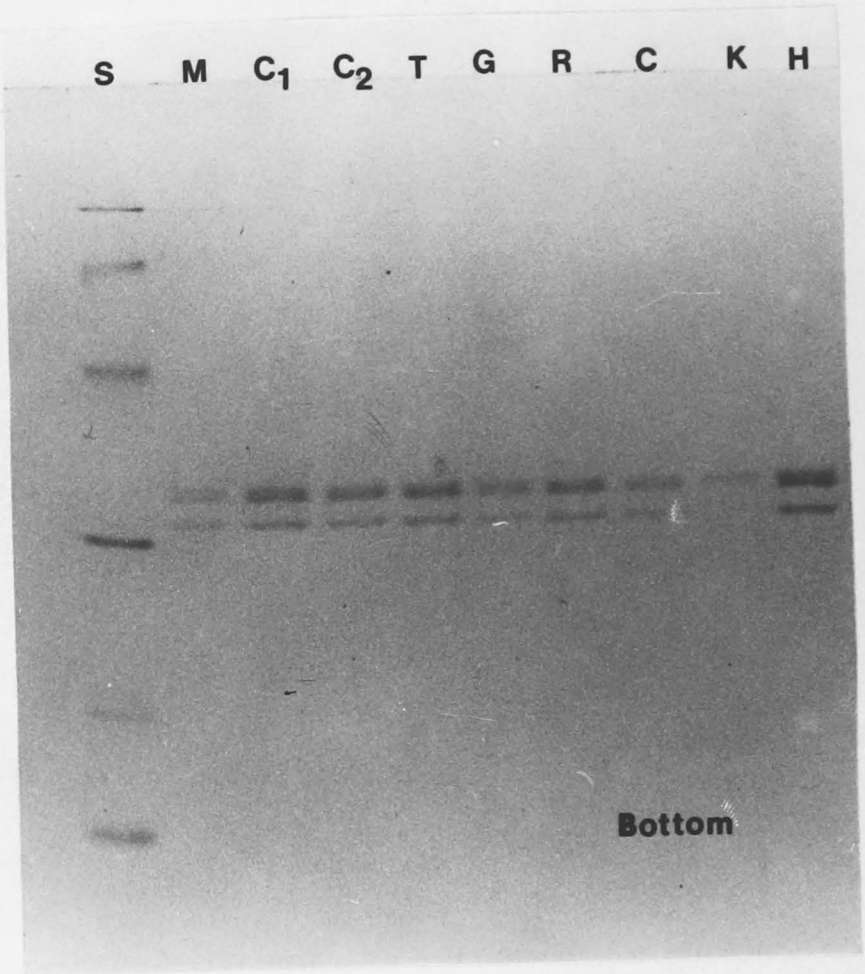
5.3.4.1 Results and Conclusions

Particle proteins from each of the 8 SBV isolates separated into 3 distinct bands in 15% SDS-polyacrylamide gels. The estimated molecular weights of each protein band (calculated from 6 separate gels, Figure 5.5) were 31,500 (± 150), 34,500 (± 150) and 35,000 (± 150) daltons, and thus were greater than those reported for SBV by Bailey and Woods (1977a) (25,000, 28,000 and 31,000 daltons). The differences are discussed further in the general discussion at the end of this chapter.

It was interesting that the particles of the two electromorphs of the Canberra isolate also resolved into three distinct protein bands in the gels, and that their

FIGURE 5.5 Coat proteins of SBV particle isolates from Millicent (M), Canberra (electrophoretic Rø value of 0.22 - C_1), Canberra (Rø value of 0.51 - C_2), Tamworth (T), Grafton (G), Richmond (R), Coonabarrabran (C), Kuranda (K) and Herberton (H).

S : Standard marker proteins (see Chapter 2).


S
M
C1
C2
T
G
R
C
K
H
Bottom

estimated molecular weights were the same as those of the other isolates. These results show that the possible proteolytic changes, perhaps caused to SBV particles by contaminating enzymes (Section 5.3.2.3), do not severely affect the size of the proteins.

Because no differences in coat protein molecular weights were found, it was considered worthwhile to determine whether there were differences in the genomes of SBV isolates. The methods described in Section 2.7 were used to obtain restriction fragment spectra of SBV genomes using the restriction endonucleases Alu I, Hae III, Hha I and Sal I to digest random and T_{12}-T_{18} oligonucleotide primed cDNA/SBV-RNA hybrid duplexes. Preliminary tests however, showed that these enzymes did not cleave the hybrids, and so I did not pursue this research any further. However, during these preliminary tests, experiments were done to determine the size of undigested SBV-RNA, and this is discussed together with similar studies using KBV-RNA in Section 5.4.3.3.

The results of my studies of strain variation using SBV isolates are discussed at the end of this chapter.

5.4 STUDIES OF STRAIN VARIATION USING KBV ISOLATES

Seven isolates of KBV were used in these studies. They were obtained from :

1) Inapparently infected pupae collected from colonies located at Canberra, Narrandera and Warwick. The

particles were extracted 3 days after inoculation with 10 µl of PP buffer. Then, extracts of the buffer injected pupae were injected into groups of healthy pupae (obtained from a colony in Canberra), and incubated for 3 days at 35°C. The pupae were then stored at -20°C until needed.

2) Moribund pupae collected from a colony at Sydney, NSW, and from an unknown location in Queensland, kindly supplied by Mr M. Hornitzky, Veterinary Research Station, Glenfield, NSW.

3) Moribund pupae kindly supplied by Mr D. Dall, Waite Agricultural Research Institute, Glen Osmond, SA.

4) Dead adult worker bees collected from the entrance of a colony at Keith, SA, by Mr L. Lacey, South Australian Department of Agriculture.

5.4.1 Gel Diffusion and Electrophoretic Mobility Tests

Individual preparations of KBV were obtained from individual pupae or adult worker bees from the localities described in Section 5.4. The serological relationships of the particles from each isolate and their electrophoretic mobilities were compared as described for SBV particle isolates (Sections 5.3.1; 5.3.2).

5.4.1.1 Results and Conclusions

The precipitin bands obtained in pairwise gel diffusion tests of each KBV isolate were completely

confluent, and hence the particles from each locality were serologically indistinguishable. However, an extract obtained from an individual adult worker bee from Keith produced two bands in gel diffusion tests; both of these bands fused with the single precipitin band formed by each of the other KBV isolates. Further tests showed that the bands were not formed by a reaction between host protein and a host specific antibody in the KBV antiserum. Furthermore, the mobilities of the particles which gave two bands were much faster than the mobilities of the other particle isolates. However, when young pupae were inoculated with the Keith preparation, the 'progeny' KBV particles had electrophoretic mobilities identical to those of the other KBV isolates. It was also noted during these inoculation experiments that preparations containing the particles with the faster mobility were not as infectious as those of the other KBV isolates. To test whether both bands observed in the original gel diffusion tests contained RNA (i.e. were intact virus particles), a gel containing both bands was soaked in 0.2% ethidium bromide, which stains only RNA or DNA. The results showed that only the band farthest from the antibody well contained RNA. These results suggest that the KBV particles with the faster mobility may have been damaged.

As no serological or electrophoretic mobility differences were found it was decided to look for differences by comparing the size of the capsid proteins from the KBV isolates.

5.4.2 Molecular Weights of Coat Proteins of KBV Particle Isolates

The same seven KBV isolates used in gel diffusion and electrophoresis tests (Section 5.4.1) were used. However, group preparations were obtained of each isolate after their particles were propagated in young bee pupae from a colony in Canberra.

The molecular weights of the KBV capsid proteins were estimated by the methods described in Section 2.6.

During preliminary experiments, I frequently found that the sizes of the KBV capsid proteins varied. This finding was also reported by Bailey *et al.*, (1979) who assessed the molecular weights of the coat proteins of other Australian KBV isolates. They suggested that this may have been due to the action of contaminating proteolytic enzymes. Therefore, the effects of freezing, and different storage temperatures on the stability of KBV coat proteins were examined. At the conclusion of these experiments, the purification method likely to yield the most stable particles was used to prepare preparations of each of the KBV isolates for PAGE of their proteins.

5.4.2.1 Experiment 1

The aim of this experiment was to determine whether the molecular weights of KBV capsid proteins in SDS-PAGE

gels were affected by freezing the KBV infected pupae before purification or by freezing preparations immediately after purification.

Particles of the Canberra KBV isolate were injected into two groups of 30 pupae. After two days incubation at 35°C, one group was removed and frozen at -20°C for 24 hours. A group preparation was obtained immediately from the other pupae, and later from the frozen pupae, and each was tested for the presence of virus particles by gel diffusion and electron microscopy as described previously.

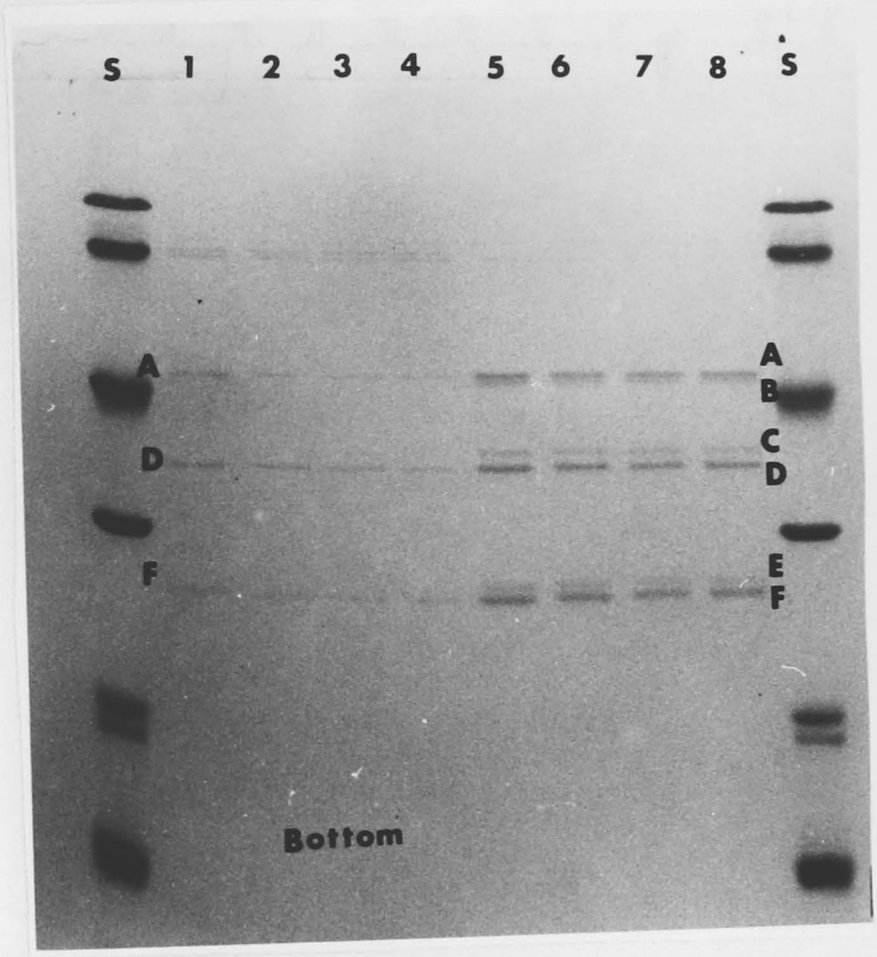
Each purified virus preparation was divided into 4 aliquots. One aliquot was frozen in liquid nitrogen, then thawed twice; another was frozen and thawed 8 times; the third frozen and thawed 16 times; the fourth aliquot was not frozen. Finally, the molecular weights of the capsid proteins of the KBV particles in each aliquot were estimated.

5.4.2.2 Results and Conclusions of Experiment 1

KBV particles purified from pupae that were not frozen prior to purification produced, in PAGE, three polypeptides in approximately equal amounts and with estimated molecular weights from 4 independent experiments of 44,500 (± 250), 37,500 (± 250) and 26,000 (± 250) daltons and these I call A, D and F respectively (Figure 5.6). Bands with identical molecular weights were obtained when these preparations were frozen and thawed 16 times (Figure 5.6). These

FIGURE 5.6 Protein bands produced by particles of a Canberra isolate of Kashmir bee virus that were either frozen (lanes 1-4) or not frozen (lanes 5-8) prior to purification. After purification the particles were either not frozen (lanes 1 and 5) or frozen and thawed in liquid nitrogen twice (lanes 2 and 6), eight times (lanes 3 and 7) and sixteen times (lanes 4 and 8).

S : Standards (details in text).


S 1 2 3 4 5 6 7 8 S
A
D
F
A
B
C
D
E
F
Bottom

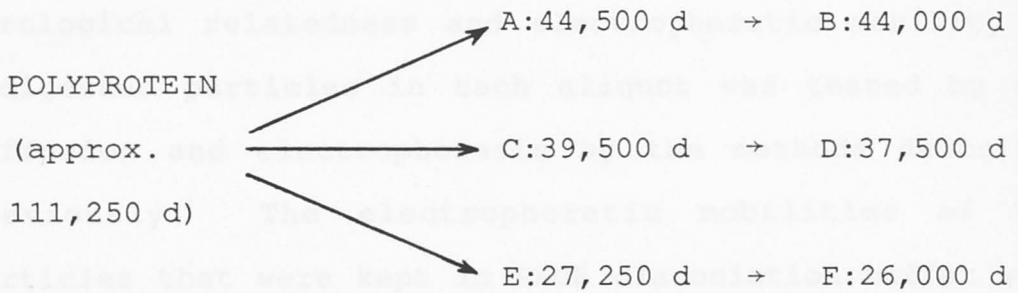
preparations also produced two minor bands with estimated molecular weights of 66,500 and 67,000 daltons. These were probably undissociated or uncleaved KBV capsid proteins.

KBV particles from pupae frozen before purification also produced the components A, D and F. However, these particles also yielded three additional protein components with estimated molecular weights, calculated from 4 independent experiments to be 44,000 (± 150), 39,500 (± 150) and 27,250 (± 150) daltons, and these I call B, C and E respectively. Furthermore, the molecular weights of these 6 components did not change when their intact particles were frozen and thawed up to 16 times (Figure 5.6).

The simplest explanation of these patterns is that the components B, D and F in Figure 5.6 were derived from the polypeptides A, C and E respectively, perhaps by degradation by proteolytic enzymes present in the preparations. If so, KBV particles probably have 3 major proteins, shown as bands A, C and E in Figure 5.6. When KBV is purified from pupae which have recently died (i.e. have not been frozen before purification), they yield the proteins seen as band A, and the degradation products of bands C and E, that is, bands D and F. Perhaps the large molecular weight bands are hetero-dimers of bands C and E, as the sum of the molecular weights of these two bands is 66,750 daltons, which is close to the estimated molecular weights for the large molecular weight bands of 66,500 and 67,000 daltons. It is interesting that preparations of

particles purified from pupae frozen prior to purification produced bands C and E, but did not produce the high molecular weight bands near the top of the gel (Figure 5.6).

The possible pathways of degradation of KBV capsid protein are summarized as follows:



To check that the results described above were reproducible, I repeated the tests using freshly passaged preparations of the particles of the Canberra and Warwick KBV isolates and obtained identical results.

5.4.2.3 Experiment 2

The aim of this experiment was to determine whether stored purified preparations of KBV changed the molecular weights of their capsid proteins.

Two groups of 30 pupae were injected with inocula from a Canberra or a Warwick KBV isolate. After 3 days incubation at 35°C, a group preparation was obtained immediately from each by the methods described in Section 5.4.2.1 (i.e. pupae were not frozen prior to purification).

Each purified extract was then divided into 5 aliquots: one aliquot was kept at room temperature; one at 4°C; one at -20°C; one was mixed 1:1 with glycerol and kept at 4°C; another was mixed 1:1 with a dissociation buffer (Section 2.6) and kept at 4°C. Then, one, seven and fourteen days later, the molecular weights of the capsid proteins in each were estimated. At the same time, the serological relatedness and electrophoretic mobility of undigested particles in each aliquot was tested by gel diffusion and electrophoresis by the methods described previously. The electrophoretic mobilities of the particles that were kept in PAGE dissociation buffer were not measured.

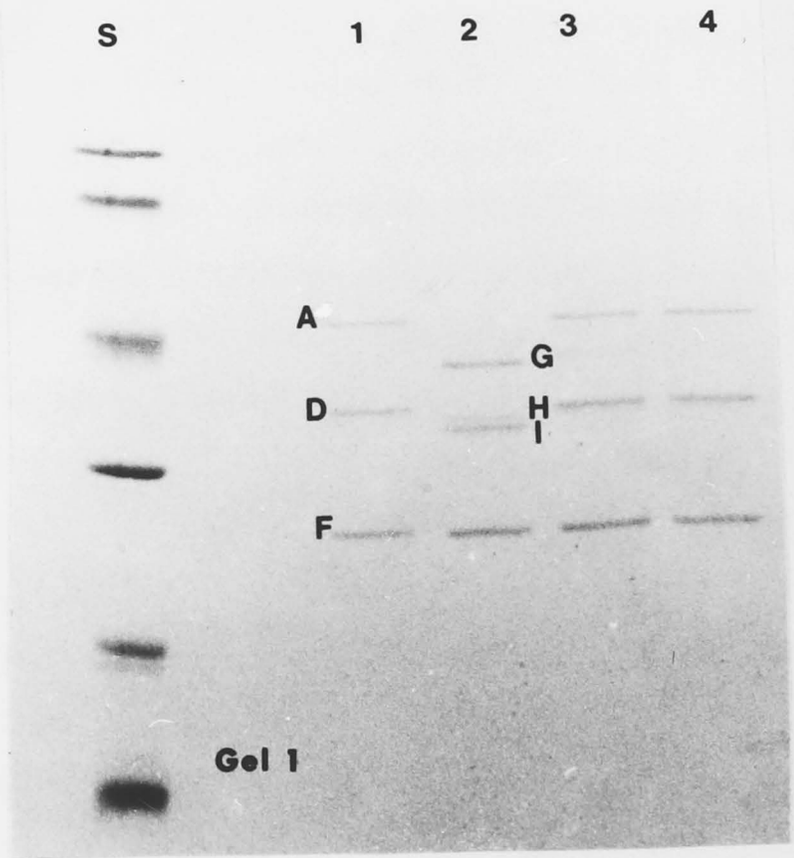
5.4.2.4- Results and Conclusions of Experiment 2

Both the Canberra and Warwick KBV isolates which were stored for one day at room temperature, 4°C, -20°C, in glycerol at 4°C, and in PAGE dissociation buffer at 4°C, produced 3 components in approximately equal amounts. The molecular weights were estimated from four experiments to be 44,500 (± 250), 37,500 (± 250) and 26,000 (± 250) daltons. These correspond to bands A, D and F in Figure 5.6.

After seven days at room temperature, the particles of both isolates produced four protein peptides (Figure 5.7). Three were different and one similar to those produced when these particles were tested after standing for one day at

FIGURE 5.7 Protein bands produced in a 15% SDS-PAGE gel by particles of a Canberra isolate of KBV that were stored for 7 days at -20^{o}C (lane 1), at room temperature (lane 2), at 4^{o}C (lane 3), and in glycerol at 4^{o}C (lane 4).

S : Standard marker proteins (see Chapter 2).


S
1
2
3
4
A
G
D
H
I
F
Gel 1

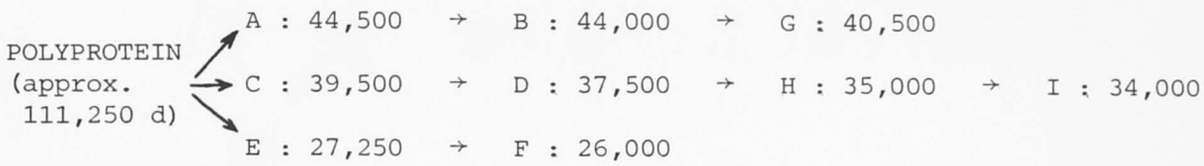
room temperature. The molecular weights of the four polypeptides were estimated from five experiments to be 26,000 (± 250), 34,000 (± 250), 35,000 (± 250) and 40,500 (± 250) daltons. The 26,000 dalton polypeptide corresponds to band F in Figure 5.6. The 34,000 and 35,000 dalton polypeptides (bands called I and H in Figure 5.7) are probably further degradation products of band C of Figure 5.6, and the 40,500 dalton polypeptide (band G in Figure 5.7) is probably further degradation of band A in Figure 5.6. Furthermore, the particles that produced these new components had a faster electrophoretic mobility in agarose gels and they produced two precipitin bands in gel diffusion tests after 14 days storage; however, when stained with 0.2% ethidium bromide, it was evident that only the precipitin band farthest from the antibody well contained RNA. These results support the conclusion in Section 5.4.1 that the particles of the original Keith KBV isolate, obtained from dead worker bees and which produced two precipitin bands in gel diffusion tests, were damaged.

Bands A and D (Figure 5.7), produced by the particles of each isolate stored at 4°C for one day, were almost completely replaced by the bands G, H and I by day 14. Furthermore, the electrophoretic mobility of these particles had become faster, and was now the same as the particles stored at room temperature. In addition, the particles were serologically indistinguishable in gel diffusion tests from the other stored KBV particles.

The changes that occurred in the capsid proteins of KBV particles were perhaps caused by contaminating proteolytic enzymes, and their source could be determined in future studies.

5.4.2.5 Summary of Studies of KBV Coat Proteins

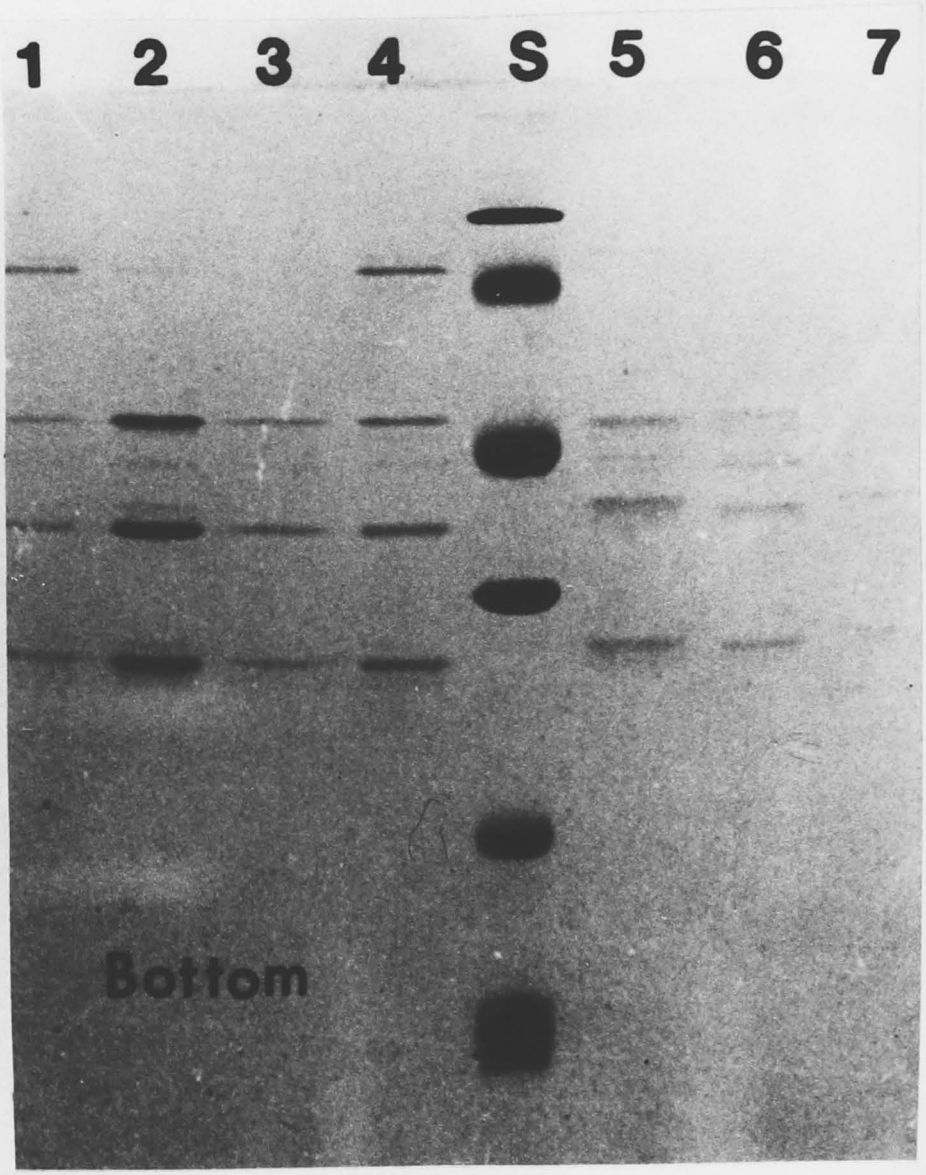
The results of Experiments 1 and 2 suggest that KBV particles contain 3 major coat proteins. The estimated molecular weights of these proteins were 44,500, 39,500 and 27,250 daltons. These proteins may be degraded into proteins of lesser molecular weight. The heaviest major protein (band A in Figure 5.6) may be degraded to a protein of 44,000 daltons (band B in Figure 5.6) which, in turn, may be cleaved to give a protein of 40,500 daltons (band G in Figure 5.7). The second heaviest major protein of 39,500 daltons (band C in Figure 5.6) may be degraded to a protein of 37,500 daltons (band D in Figure 5.6) which is subsequently digested to proteins of 35,000 or 34,000 daltons (Bands H and I in Figure 5.7). The lightest major protein of 27,250 daltons (band E in Figure 5.6) may be degraded to a protein of 26,000 daltons (band F in Figure 5.6); this protein appears quiet stable. The possible pathways of degradation are summarized as follows:



The results of Experiments 1 and 2 show that some degradation of the 3 major capsid proteins of KBV occurred during preparation and storage. However, they suggest that KBV particles with the 3 major capsid proteins (bands A, C and E in Figure 5.6) might be obtained by inoculating young bee pupae, incubating them for 2 days, then freezing them for 24 hours before purifying the KBV particles in them, and immediately testing them by PAGE. Therefore, particles from such material were used for determining the molecular weights of the capsid proteins of the other KBV isolates. Figure 5.8 shows the polypeptides produced by each KBV particle isolate using this method, and it is apparent that some of the major capsid proteins were still degraded, suggesting that additional methods may be required to inhibit the degradation of purified KBV particles. However, even this may not necessarily yield unambiguous results of value for distinguishing between KBV isolates. A more satisfactory way of differentiating KBV isolates may be by analyzing the genomes directly.

FIGURE 5.8 The protein components produced in a 15% SDS-PAGE gel by particle isolates of Kashmir bee virus from Canberra (1), Narranderra (2), Adelaide (3), Keith (4), Warwick (5), Sydney (6) and from an unknown location in Queensland (7).

S : Standards (details in text).


1
2
3
4
S
5
6
7
Bottom

5.4.3 Preliminary Analysis of the Genomes of KBV Particle Isolates

Recently it has been shown that RNA viral genomes can be usefully compared in a semi-quantitative way by examining the fragments obtained:

1) When the genome is hydrolysed by a site-specific endonuclease such as T1 ribonuclease, and the resulting oligonucleotides separated by two dimensional chromatography to give a 'fingerprint' pattern (Kew *et al.*, 1981); and,

2) When the genome is transcribed by reverse transcriptase to give a cDNA/RNA hybrid, and this is cut by restriction endonucleases to give fragments that are separated according to their sizes in PAGE (Faragher *et al.*, 1985).

I decided to compare the genomes of KBV isolates using the cDNA/RNA restriction enzyme method, and report here some preliminary results.

The enzyme Hind II was the first restriction endonuclease found that would cleave a double-stranded (ds) DNA molecule at a specific site (Smith and Wilcox, 1970). Since its discovery, many other restriction enzymes which cut dsDNA molecules have been isolated. Most of these recognize specific palindromic sequences of 4 or 6 bases in each chain of the molecule. Obviously, those that bind to

4 bases are less specific than those that recognize 6 bases and hence usually cut DNA molecules at more sites.

Danna and Nathans (1971) reported that Hind III cut circular dsDNA of simian virus 40 into specific fragments that were easily separated (depending on their length) by electrophoresis to form a specific 'restriction fragment spectrum' (RFS). Blakesley and Wells (1975) used Hae III to specifically cleave 'single-stranded' (ss) DNA from the bacteriophage ϕX174 and M13, and Molloy and Symonds (1980) demonstrated that a number of restriction enzymes recognized specific base sequences in cDNA/virus-RNA hybrids and faithfully cut at least the cDNA strand of the hybrid.

RFS's and restriction maps, in which the restriction 'fragments' have been unequivocally aligned, are nowadays widely used to distinguish between closely related DNA or RNA virus genomes. The sensitivity of the method depends upon the length of sequence recognized and the number of such sequences in the nucleic acid being studied. Thus a restriction site of 4 nucleotides represented 25 times in a genomic nucleic acid represents a total of 100 nucleotides in the genome. A difference of 1 nucleotide would change 2 fragments in the RFS, and if the restriction sites were an unbiased sample of the whole nucleic acid, such a change would represent a 1% difference. Sequencing the bases of the viral genome is more sensitive, but this technique is both time consuming and expensive.

5.4.3.1 KBV Isolates and RNA Extraction

The 7 KBV isolates used for KBV coat protein analysis (Section 5.4.2.) were studied. Virus particles from each isolate were replicated in groups of 30 young bee pupae, purified, and the RNA extracted as described in Chapter 2.

5.4.3.2 The Experiments

1) Determining the Size of Undigested RNA - The molecular weights of undigested KBV RNA, extracted from a Canberra isolate, and undigested SBV ssRNA from a Canberra and a Kuranda isolate were estimated by the methods described in Section 2.7.2 using CrPV RNA as a standard.

2) Restriction Enzyme Analysis of cDNA/KBV Hybrids - Hybrids were prepared and digested as described in Section 2.7.3.

An experiment was done initially to determine how many fragments were produced in gels when random or T_{12}-T_{18} oligonucleotide primers were used, and the resulting cDNA/KBV-RNA hybrid duplexes digested with Alu I, Hae III, Hha I or Sal I enzymes (Chapter 2). The enzyme that produced the largest number of fragments was used to obtain RFS's of the genomes of each of the KBV isolates. Then, the RFS's were compared.

Although I had no cDNA markers of known molecular weights with which to estimate the sizes of the fragments found in my PAGE experiments, the conditions used were

close to those of Faragher *et al.*, (1985) and thus may be extrapolated in a general way from their RFS's, which included oligonucleotides they had sequenced and of which they knew the sizes.

5.4.3.3 Results and Discussion

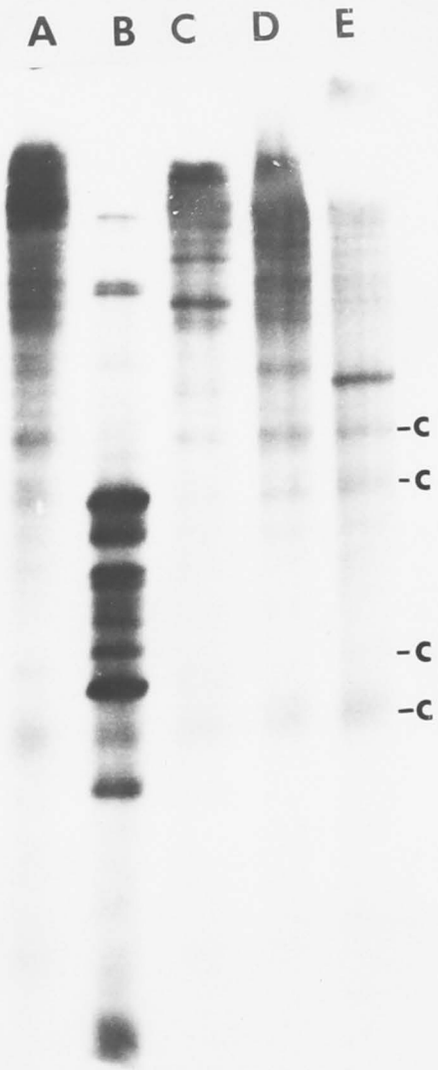
The RNA isolated from the single KBV isolate, and from each of the two isolates of SBV, migrated as a single band at a rate that was indistinguishable from that of CrPV RNA. Hence, KBV and SBV genome RNA has the same molecular weight as that of CrPV, reported to be 2.8×10^6 daltons by Eaton and Steacie (1980).

The cDNA product of randomly primed KBV genome RNA was a series of small fragments, ranging in size from approximately 100 to 300 bases in length, and no additional bands were produced as a result of treatment with either of the four restriction enzymes. These results, combined with those obtained using T_{12}-T_{18} primers, suggest that the fragments were all from parts of the genome characterized by a lack of restriction sites; possibly all the fragments resulted from initiation of cDNA synthesis at a single site, but terminated at various sites.

By contrast, when T_{12}-T_{18} was used to prime cDNA synthesis on the KBV genome template, larger cDNA's were obtained and most of the radioactivity was incorporated in a range of molecules approximately 200-600 nucleotides long (Figure 5.9). Treatments of this product with the four

FIGURE 5.9 An autoradiograph of a 4% polyacrylamide gel showing fragments produced using $T_{12 - 18}$ primed cDNA/KBV RNA hybrid duplexes (Keith isolate) that were not treated with restriction endonucleases (A), or were treated with the endonucleases Alu I (B), Hae III (C), Hha I (D) or Sal I (E).

-c : Fragments common to each treatment.


A
B
C
D
E
-C
-C
-C
-C

restriction endonucleases gave RFS's that were mostly different; only four fragments (labelled -c in Figure 5.9) were common to 3 or more of the digests. The restriction enzyme which gave the RFS most distinctly different from that of the undigested cDNA was Alu I. The Alu I RFS had 8-12 bands of approximately equimolar amounts ranging in size from about 75-150 nucleotides. This indicates that there are probably 7-11 Alu I restriction enzyme sites in the KBV cDNA. Thus this enzyme interacted with 28-44 nucleotides in the genome, and hence would detect a minimum average sequence difference of 2-4%.

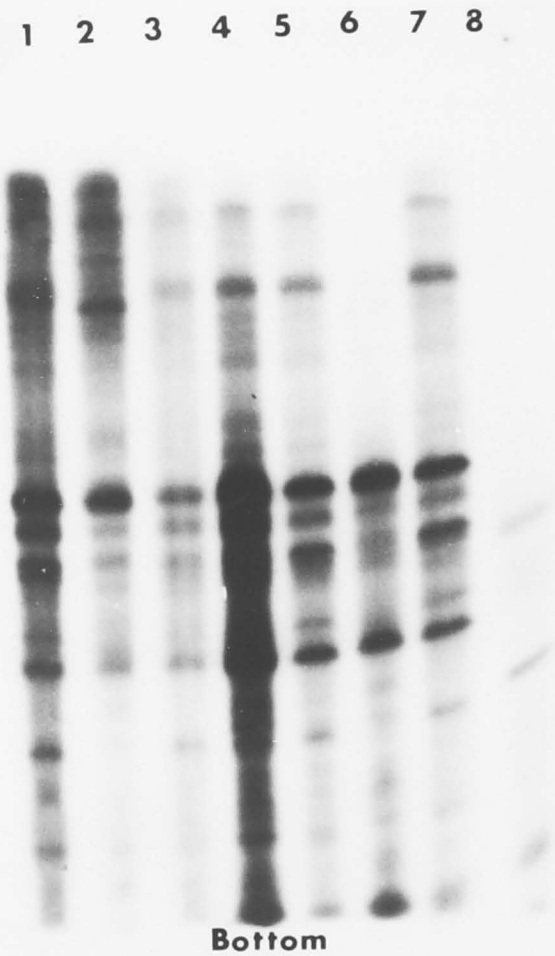
Figure 5.10 shows an autoradiograph of an acrylamide gel comparing the RFS's produced by Alu I with cDNA/RNA hybrids prepared from the genomes of each of the KBV isolates. The RFS of the Keith KBV isolate is shown in Figures 5.9 and 5.10 (Lane 1). The RFS's shown in Figure 5.10 are clearly mostly similar, though there are some differences in density of the major bands and different minor bands, which suggest that this method has potential for distinguishing between KBV isolates. However, it is clear that more work is needed to clarify these results and assess the potential of Alu I for detecting differences between KBV isolates for use in ecological studies.

5.5 DISCUSSION

Despite extensive studies using SBV isolates from widespread localities, no reproducible strain differences

FIGURE 5.10 An autoradiograph of a 4% polyacrylamide gel showing fragments produced using the restriction endonuclease Alu I to digest cDNA/KBV RNA hybrid duplexes from the genomes of KBV particle isolates from Keith (1), Narranderra (2), Sydney (3), Adelaide (4), Canberra (5), Warwick (6), Canberra* (7), and from and unknown locality in Queensland (8).

* These hybrid duplexes were prepared independently from the same KBV RNA used to prepare the hybrid duplexes used in lane 5.


1 2 3 4 5 6 7 8
Bottom

were detected. This may have been because relatively insensitive methods such as gel diffusion, isoelectric focussing and relative electrophoretic mobility of particles and proteins (SDS-PAGE) were used for most of the isolates. Future tests for variation amongst SBV isolates may be directed at using more sensitive techniques such as restriction enzyme mapping or sequencing of the SBV genome. The apparent lack of variation amongst SBV isolates will be further discussed in the following chapter.

It is noteworthy that the molecular weights of the coat proteins of SBV particles found in my studies, and in those reported in an earlier study (Anderson, 1980), are greater than those reported by Bailey and Woods (1977a). These conflicting reports may reflect different laboratory conditions under which the tests were done. Alternatively, the weights reported by Bailey and Woods (1977a) may either be incorrect or their SBV isolate was different from those I tested.

Bailey *et al.*, (1979) reported three strains of KBV from Australia that differed from one another and from an isolate from Kashmir by the size and stability of their coat proteins and in their reactions in gel diffusion tests. All the KBV isolates used in my studies were serologically indistinguishable. My studies also showed that the size of coat proteins is an unreliable determinant for characterizing KBV isolates, as each coat protein is unstable and may be degraded into one or more smaller proteins. Therefore, it would be necessary to determine

all the degradation products that are produced from each major protein of each particle isolate before differences in the coat proteins were used as a means of characterizing isolates; or, alternatively, it would be necessary to find methods to overcome the effects of proteolytic enzymes. The reasons why no distinct strains of KBV were detected in my studies are discussed in the following chapter.

The preliminary results obtained when comparing RFS's of KBV isolates show the potential this method may have for detecting differences between KBV isolates in future ecological studies.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The work reported in this thesis covers several areas of honey bee pathology which have previously received little or no attention in Australia. My studies commenced by surveying the occurrence and distribution of bee viruses in colonies of the native bee, *Trigona*, and in eastern Australian honey bee colonies. Initially, methods for studying bee viruses in other countries were used, but it soon became obvious that they were giving ambiguous results because several viruses existed inapparently in Australian bees and these were often activated during experimental work. Not even the method of propagating bee viruses in young bee pupae (Bailey and Woods, 1977b), which is used by most overseas bee pathologists, could be used as this often activated inapparent virus infections and hence contaminated preparations were produced. Therefore, methods for obtaining uncontaminated virus preparations had to be devised so that specific antisera could be produced and ecological and characterization studies undertaken. Initial attempts to find alternative systems for propagating bee viruses were fruitless, and thus it became evident that research could only proceed by studying inapparent infections. From these studies new methods for

manipulating bee viruses were developed which will be useful for future research. However, some intriguing results from my studies of KBV and SBV are worthy of further discussion and consideration in the general framework of what is known about these viruses.

6.1 SACBROOD VIRUS

The general picture of SBV in Australia seems similar to that in bee colonies in England, even though the colonies are subject to different climatic and environmental conditions (Bailey, 1981; Hornitzky, 1982). For example, sacbrood disease is common in Australian bee colonies in spring and early summer (Chapter 3), as it is in England, and biophysical and biochemical tests suggest that Australian SBV isolates are indistinguishable from each other (Chapter 5) and from those in the United Kingdom (Bailey, 1982). Indeed, all isolates of SBV obtained from honey bees throughout the world appear to be indistinguishable, though a distinctly different strain has been isolated from *Apis cerana* from Thailand (Bailey, *et al.*, 1982). There are many possible reasons for this apparent uniformity among honey bee SBV isolates. Perhaps the methods I and others have used were insufficiently discriminatory and it may be necessary, for example, to sequence parts of their genomes to detect variation. Alternatively, there may be very little variability, either because the virus has only recently spread through the bee

population of the world, or because it has co-evolved with *A. mellifera* for a long time, and all variants that have arisen have been lost as a result of strong selection for one type, the strain now found worldwide.

The two most important pathogens of larvae in Australian honey bee colonies are the bacteria *Bacillus larvae* and *Melissococcus pluton*, which cause American and European foulbroods respectively (Hornitzky, 1985). Sacbrood disease is considered by apiarists to be the third most important pathogen of larvae because in some colonies it causes considerable mortality in the larvae. However, the results of my studies suggest that in addition to the noticeable damage caused by SBV to late instar larvae, there should also be added the losses of early instar larvae and the less noticeable effects caused to adult worker bees by inapparent SBV infections. In England, inapparent SBV infections have been shown to prematurely age adult worker bees and to have detrimental effects on their collecting and foraging ability (Bailey and Fernando, 1972; Fernando, 1972). In those studies it was shown that adult bees became infected with SBV when 'house cleaning' as young bees (see Chapter 1), but in my studies, adult bees were shown to become persistently infected after eating contaminated food as larvae. It is not known whether adult bees which are infected as larvae prematurely age like those infected as adults, however if they do, then the unnoticed effects of SBV on adult bees in Australian colonies may considerably add to the damage that SBV

occasionally causes by killing late instar larvae. For this reason, inapparent SBV infections in Australian colonies may be economically important. Alternatively, inapparent infections acquired by bees from the larval stages may affect adult bees differently. It would be worthwhile to study these possible differences in the future, and to attempt to locate the sites of inapparent SBV infections in larvae, pupae and adult bees.

It is interesting that inapparent SBV infections of bee pupae have not been reported in England where the life cycle of SBV has been well studied (see Chapter 1). If inapparent SBV infections do occur in English bee pupae they probably behave differently than in Australia, as it is extremely unlikely that such easily activated infections would have remained unnoticed. On the other hand, the presence of inapparent SBV infections in Australian bee pupae may reflect a response by SBV to different selection pressures, possibly exerted by microorganisms not encountered in English colonies, such as KBV, or perhaps by stresses caused by different beekeeping practices. For example, beekeeping in Australia differs from that in England in that it is mainly migratory, with beekeepers following 'honey flows'. In this way, many Australian bee colonies are frequently subjected to changes in climatic and environmental conditions and this probably stresses the colonies.

Sacbrood disease is common in bee colonies in eastern Australia during spring and early summer (Chapter 3;

Hornitzky, 1985). At this time of year, colony populations rapidly expand due to their increased larval production, as pollen and nectar become more abundant and the environmental temperatures increase. During these 'spring build-ups', there are usually not enough young nurse bees to attend the increasing numbers of developing larvae, and thus older bees, which have usually been present in the colonies during the winter months, resume rearing duties (Kauffeld, 1980). Bailey (1969) suggested that many 'overwintering' worker bees are inapparently infected with SBV and they probably initiate spring outbreaks of sacbrood by contaminating the larval food with SBV particles. Later in the spring, when the proportion of young nurse bees within colonies increases, and the division of labour also becomes well established, there is less liklihood of older infected bees contacting young developing larvae and hence sacbrood becomes less common. However, the seasonal occurrence of sacbrood may be more complex than this, as my results showed that not all larvae die when fed SBV particles, but that large proportions become inapparently infected with the virus. Therefore, it is likely that a proportion of larvae fed contaminated food by 'overwintering' bees develop persistent SBV infections. This hypothesis is supported by my results which showed that the incidence of inapparent SBV infections increased in a normal colony during spring and early summer (Chapter 4). If larvae do acquire inapparent SBV infections from 'overwintering' bees, then the reasons why SBV contaminated

food kills some larvae and causes others to become inapparently infected are not fully understood. My work showed that the age of larvae and the concentration of SBV particles contaminating the food affects the proportions of larvae that become inapparently infected, however, the genetic susceptibility of larvae may also be important.

Very little is known about inapparent virus infections within insect populations but their common occurrence suggests they probably fulfill an important role in virus persistence. Their incidence in bee colonies provides a reservoir of material for future studies.

6.2 KASHMIR BEE VIRUS

This virus is not known to occur in honey bees outside Australia and very little is known of its ecology. During this study, attempts to find KBV in colonies of the social bee *Trigona*, which is closely related to the honey bee, were unsuccessful (Chapter 3), suggesting that KBV may be confined to honey bees and is not a pathogen of another closely related insect from which it occasionally spreads to honey bees, as was suggested by Bailey *et al.*, (1979) and Bailey (1981). Dall (1985) also suggested that KBV in Australian bees was unlikely to have been acquired from another insect species.

Bailey *et al.*, (1979) isolated several serologically distinct strains of KBV from Australian honey bees, and Bailey (1981) suggested that the presence of these strains

may reflect a process of mutation and selection that is still being undergone by KBV as it adapts to the honey bee in Australia. It was interesting therefore that no serologically distinct isolates of KBV were detected during my work, despite testing KBV isolates from several widespread localities. This may suggest that the KBV antiserum used in my work was not as discriminatory as that used by Bailey *et al.*, (1979). Nevertheless, the unstable nature of KBV coat proteins, as demonstrated in my work (Chapter 5), suggests that the results from serological tests using KBV antiserum should be regarded with caution. For example, if partially degraded particles are used to produce an antiserum, novel antigenic determinants may be exposed, and hence such particles may produce an antiserum which appears to be unique. Less ambiguous results might be obtained in future studies by cloning segments of the KBV genome and comparing sequence differences between isolates. Such techniques would also enable specific probes to be obtained which may be useful in ecological studies.

During my work, and in studies by Dall (1985) and Hornitzky (1985), inapparent KBV infections were found to be common and widespread in Australian colonies. Therefore, in the past, KBV has probably been exported in live bees from Australia to other countries. Nevertheless, KBV has not been reported from other countries possibly because research workers have not looked for inapparent virus infections in their bees. Alternatively, the

climatic and environmental conditions of some countries may eradicate the virus, and this may explain why KBV has not been detected in English colonies (Bailey, 1981) even though England has imported live bees from Australia.

I found that the particles of KBV were unstable in purified preparations and quickly lost their infectivity in dead adult bees collected from the entrances of colonies. This suggests that KBV does not survive well outside the host and may require contact between live infected individuals to spread. Thus, adults infected as larvae may contaminate larval food and be an important vehicle for long-term persistence of the virus in colonies.

The site of inapparent KBV infections in bees is not known, but one possible site is the cells lining the gut. When bee larvae pupate they expel the gut contents, but the gut lining is not expelled and becomes part of the pupa and later, the adult bee (Snodgrass, 1956). Thus, by infecting the gut lining of larvae, KBV might have a vehicle by which to infect individuals at other developmental stages. Interestingly, Bailey and Woods (1977b) found that adult worker bees did not die when fed KBV, but did when KBV was injected into their haemocoels. This suggests that the gut of worker bees may be a barrier to KBV infection, and when it overcomes this barrier (i.e. by injection directly into the haemocoel), the virus rapidly grows and kills the bee. In future studies it would be interesting to test whether adult bees can establish inapparent KBV infections when fed contaminated food, like they do as larvae (Chapter 4).

If the gut is a site for inapparent KBV infections, then in reported cases of KBV killing larvae (Rhodes and Teakle, 1978) and worker bees (Bailey, 1981) in the field, the virus must have overcome the gut barrier. This might occur when the virus infects genetically susceptible bees or, alternatively, when the bee gut becomes infected with a primary pathogen. This latter hypothesis is supported by a finding which showed that KBV is often isolated from colonies in association with the bacterium, *M. pluton* (Hornitzky, 1982). This bacterium causes a gut infection in larvae which may damage the gut lining, thereby allowing KBV to infect other tissues. Furthermore, Rhodes and Teakle (1978) described symptoms similar to those caused by *M. pluton* in larvae from which KBV particles were obtained in high concentrations.

From this brief discussion it is clear that much research needs to be done on KBV in Australian honey bees. A study of the behaviour of inapparently infected adult bees may shed more light on the ecology of this virus.

6.3 CONCLUDING REMARKS

My studies suggest that in Australia KBV is confined to honey bees, contrary to the suggestion of Bailey *et al*., (1979) and Bailey (1981) that KBV is a virus of another insect species and spreads from that reservoir to the honey bee population. Thus, both SBV and KBV seem to be, specifically, viruses of honey bees. Indeed, most insect

picornaviruses may be as host specific as picornaviruses of mammals, and wide host range insect picornaviruses such as cricket paralysis virus and *Drosophila* C virus (Scotti *et al.*, 1981) may be atypical.

Reseach on the diseases which affect the honey bee is important because many diseases reduce the efficiency of bees as honey gatherers and as pollinators. Despite this, most of the virus diseases of honey bees remain poorly studied and there is not enough information available to control them effectively. Research on viruses of Australian honey bees has only recently commenced, but it is essential that it be continued, as recent results have shown that Australian honey bees have virus disease peculiarities which may be economically important.

The rapid developments recently made in molecular genetics may be used in the future to produce bees that are resistant to virus infections. Plant-virus control schemes utilizing these new methods to manipulate and move viral genes for protection (Gibbs and Skotnicki, 1986) are currently being investigated. Their success will help clarify the feasibility of similar schemes for honey bees, where transforming systems based on the transposon-like P-elements of *Drosophila* (Rubin and Spradling, 1982) may prove useful.

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APPENDIX A

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A Comparison of Serological Techniques for Detecting and Identifying Honeybee Viruses

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The sensitivity and specificity of conventional Ouchterlony gel-diffusion, immuno-osmoelectrophoresis (IO), immune serum electron microscopy (ISEM), "decoration," radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA) tests for detecting black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV), and sacbrood virus (SBV) particles in extracts of diseased honeybees were compared. A "slow" ISEM method detected virus particles in extracts of individuals or groups of individuals diluted to 10^{-3} and 10^{-4}, respectively, whereas the IO method and a "fast" ISEM method using protein A were one-tenth as sensitive, and Ouchterlony gel-diffusion tests were only one-thousandth as sensitive. Using the antibody "decoration" technique, mixtures of serologically unrelated virus particles could be resolved. RIA and ELISA were found to be one thousand times more sensitive than ISEM in detecting the particles of BQCV, CBPV, KBV, and SBV; however, nonspecific reactions occurred when using RIA with very dilute particle suspensions, and this made dilution endpoints difficult to assess, but this did not occur when using the ELISA method. There was little difference in the effectiveness of rabbit or hen antisera in the tests, except when protein A was used as it does not combine with hen antibodies.

KEY WORDS: *Apis mellifera;* honeybee viruses; serological methods of detecting virus particles.

INTRODUCTION

Bailey et al., (1963) first isolated viruses from the honeybee, *Apis mellifera,* and since then more serologically unrelated viruses have been isolated from the honeybee than from any other insect species (Bailey, 1981).

A method for isolating most viruses of honeybees has been described by Bailey et al. (1980). This method uses samples consisting of 30 or more diseased individuals and, after they have been purified and concentrated by ultracentrifugation, the virus particles in them can be seen in an electron microscope (EM) or, alternatively, the presence of virus particles can be confirmed using conventional gel-diffusion tests (Mansi, 1958).

Serological testing of honeybee viruses is difficult because many, if not most, preparations of honeybee viruses are mixtures. This is because most honeybee colonies contain one or more viruses (Bailey et al., 1981; Hornitzky, 1981; Anderson, 1983), and bee viruses are usually obtained from, or grown in, individuals from such colonies; no virus-free cultures of bee cells have been produced, and no cells from other species have been found to be susceptible to bee viruses. Therefore, it is difficult to produce truly specific antisera to each bee virus and, in ecological studies, extracts of bees often contain more than one virus. Furthermore, the sensitivity of gel diffusion and negative staining for detecting virus particles is poor and their use for testing large numbers of extracts is laborious. Bailey et al., (1981) suggested that the enzyme-linked immunosorbent assay (ELISA) method might be advantageous for detecting viruses in honeybees during surveys or where many individuals need examining. Therefore, the potential for using ELISA (Voller et al., 1980) and other

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serological methods such as immunoosmoelectrophoresis (IO) (Scotti and Wigley, 1982), immune serum electron microscopy (ISEM) (Derrick, 1973), and radioimmunoassay (RIA) (Kemp and Cowman, 1981), to detect virus particles in extracts from individual and groups of diseased honeybees was investigated.

IO, also called electrosyneresis, counter immunoelectrophoresis, immunoelectroosmophoresis, or counter electrophoresis, involves placing negatively charged antigen and its specific uncharged antibody in wells in a gel in such a way that, when a voltage gradient is applied so that it passes parallel to the line joining the center of the two wells, the antigen migrates toward the anode while the antibody is moved in the electroendosmotic flow of water in the gel toward the cathode. Thus, antigen and antibody are brought together and a precipitin line forms where they meet.

The ISEM method involves trapping virus particles on EM grids, supporting films of Formvar and carbon which have been coated with antibodies from a specific antiserum, and examining these in an EM. Shukla and Gough (1979) found that it was quicker to pretreat the EM grids with a suspension of protein A (Pharmacia) extracted from the cell walls of *Staphylococcus aureus,* as this protein specifically reacts with gamma globulins (IgG). Milne and Luisoni (1975) demonstrated that virus particles trapped by antibodies on EM grids could be even more specifically identified by further treatment with antibodies, as these produce a specific effect termed "decoration."

The less widely used RIA method involves covalently binding virus particles to cyanogen bromide (CNBr)-activated paper. Covalently bound virus particles are detected by their ability to bind specific antibodies. These in turn are detected by their ability to bind ^{125}I-labeled protein A, and this is detected by autoradiography.

ELISA is now a standard virological detection method and has been used to detect many plant and animal viruses. In the "direct ELISA" method, wells of a microtiter hemaglutinin plate are coated with IgG (or their $F(ab')_2$ fragments) from a specific antiserum. Homologous antigen in test extracts is thus trapped, and will attract and bind to added specific IgG (or their $F(ab')_2$ fragments) conjugated with an enzyme. The presence of the enzyme is detected by a color change, observed or measured, after adding specific enzyme substrate in buffer. The "indirect ELISA" method differs in that the microtiter wells may be coated with either specific IgGs or their $F(ab')_2$ fragments or antigen. Bound antigen attracts and binds to added specific IgG which, in turn, attract and bind either IgG conjugated with enzyme and which has been prepared in a second species against the Fc region of IgG molecules, or protein A conjugated with enzyme. These are then detected as in the direct ELISA method.

This paper describes how the techniques of IO, ISEM, "decoration," RIA, and ELISA were successfully applied to detect and identify virus particles in extracts of diseased honeybees. The specificity, sensitivity, advantages, and disadvantages of each technique were compared with the results obtained by gel diffusion and electron microscopy negative staining tests.

MATERIALS AND METHODS

The viruses. Four viruses of honeybees were used: sacbrood virus (SBV), black queen cell virus (BQCV), Kashmir bee virus (KBV), and chronic bee paralysis virus (CBPV). The first two were purified, by the methods described below, from diseased larvae collected from honeybee colonies in New South Wales (NSW); KBV was similarly purified from moribund pupae, obtained by injecting latently infected pupae at the white-eyed stage with 10 μl 0.01 M phosphate buffer, *p*H 6.7; CBPV was similarly purified from adult worker bees that had died of chronic paralysis disease, and which were kindly supplied by Mr. M. Hornitzky, Department of

Agriculture, Veterinary Research Station, Glenfield, NSW. The identity of virus particles in purified extracts was determined by conventional Ouchterlony gel-diffusion tests and negative staining electron microscopy, and extracts containing obvious mixtures of viruses were discarded. When mixed preparations of virus particles were required, they were made by mixing the purified extracts.

Purification. A "group preparation" was made by grinding 30 individual bees in a mixture of 27 ml 0.01 M phosphate buffer, *p*H 6.7, and 3 ml of 20 mM sodium diethyldithiocarbamate (DIECA); shaking with 3 ml of ether and then with 3 ml of carbon tetrachloride (CCl_4), and finally centrifuging at 3000*g* for 10 min. The supernatant extract was then centrifuged at 75,000*g* for 3 hr, and the sediment was resuspended in phosphate buffer and again centrifuged briefly (Bailey et al., 1980). "Individual preparations" were obtained in a similar way from single larvae, pupae, or adult worker bees by grinding each individual in equivalent volumes of the various fluids: 0.9 ml 0.01 M phosphate buffer, *p*H 6.7, and 0.1 ml 20 mM DIECA, shaken with 0.1 ml ether and then with 0.1 ml CCl_4, and centrifuged at 10,000*g* for 30 sec. These group and individual preparations were used directly in ISEM, "decoration," IO, RIA, ELISA, gel diffusion, and negative staining experiments. In addition, individual preparations were further concentrated by centrifuging at 80,000*g* in a Beckman Airfuge for 3 min, resuspending the sediment in 0.2 ml phosphate buffer, and testing for the presence of virus particles by negative staining and gel diffusion tests. These results were used to assess whether a marked increase in sensitivity was observed after concentrating individual extracts by high-speed centrifugation.

Production of antisera. Thirty individual preparations of the particles of BQCV, CBPV, KBV, and SBV were individually checked, pooled and purified as described for group preparations, and then further purified using 10–40% sucrose gradients. Antisera against each were produced in individual rabbits (one SBV antiserum was also produced in a hen) by immunizing each with three 1-ml injections of 1 mg/ml of virions over a 5-week period; the first injection was intravenous, the others intramuscular, using immunogens emulsified in Freund's complete adjuvant. Sera were obtained 1 to 2 weeks after the final injection, 0.1 mg/ml sodium azide added as preservative, and the sera were stored at 4°C. All sera were tested in gel diffusion tests with homologous virus particles before being used in experiments. The reciprocals of homologous titers are shown in Table 1.

Serological methods. Conventional Ouchterlony gel-diffusion tests (Ouchterlony, 1964) were done using 4 ml 0.75% agar or agarose in 50 mM potassium phosphate buffer, *p*H 6.7, containing 5 mM EDTA and 0.2% sodium azide on a 40 × 80-mm sheet of "Gel-bond" (Pharmacia). Tests were done using dilutions of individual and group preparations of BQCV, CBPV, KBV, and SBV particles and homologous antiserum.

TABLE 1
RECIPROCALS OF HOMOLOGOUS TITERS OF ANTISERA OBTAINED IN CONVENTIONAL OUCHTERLONY GEL-DIFFUSION TESTS AND THE RECIPROCALS OF THE TITERS OBTAINED WHEN USING THESE DILUTED SERA IN IMMUNE SERUM ELECTRON MICROSCOPY "DECORATION" TESTS

Antiserum	Reciprocals of homologous titers in gel diffusion	Reciprocals of titers in "decoration" tests
BQCV[a]	512	16
SBV[a]	32	8–16
SBV[b]	8	16–32
CBPV[a]	64	8
KBV[a]	64	8–16

Note. The antisera were produced against the particles of either black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV) or sacbrood virus (SBV).

[a] Antiserum produced in rabbits.

[b] Antiserum produced in a hen.

The reactants were placed in circular wells 4 mm in diameter in a hexagonal pattern and 2 mm apart.

IO tests were done as described by Scotti and Wigley (1982). Glass microscope slides were precoated with 3 ml 0.2% agarose in distilled water, dried, and further coated with 3 ml 1% agarose in 0.02 M phosphate buffer, *p*H 7.2. Reactants were placed in wells 5 mm in diameter and 10 mm apart, a 35 mM Tris, *p*H 8.4, running buffer was used, and a constant potential drop of ca 3 V/cm^2 was applied across the gel for 15 min. Precipitin lines were either observed immediately with the aid of a light beam, or detected after drying the gel, and staining for 3 min in 0.25% Coomassie brilliant blue, followed by destaining in methanol:acetic acid:distilled water (4.5:1:4.5). Tests were done using 10-fold dilutions of individual and group preparations of BQCV, CBPV, KBV, and SBV particles, and specific rabbit antiserum used at their gel diffusion titers (Table 1).

The ISEM method described by Derrick (1973) will be referred to as the "slow" ISEM method, and the ISEM method described by Shukla and Gough (1979) will be referred to as the "fast" ISEM method.

For the slow ISEM method, carbon-coated Formvar films supported on EM grids were floated on 50-μl drops of specific antiserum for 30 min, then washed with 20 drops 30 mM phosphate buffer, *p*H 7, floated on 50-μl drops of suspensions of virus particles overnight at 4°C, washed, negatively stained with 20 drops 2% ammonium molybdate in distilled water, dried with fine strips of filter paper, and examined in the EM.

For the fast ISEM method, Formvar/carbon films on EM grids were floated on 50-μl drops of protein A solution (0.1 mg/ml) for 10 min, then on 50-μl drops of specific antiserum for 30 min, washed, floated on 50-μl drops of suspensions of virus particles for 20 min, washed, negatively stained, dried, and examined in the EM. Experiments were done using twofold dilutions of specific antisera to determine which dilutions trapped the most virus particles using the fast and slow ISEM methods. Control grids were prepared using sera obtained from nonimmunized rabbits and hens. The sera were diluted in a buffer consisting of 1.59 g/liter disodium carbonate and 2.93 g/liter sodium hydrogen carbonate ($NaHCO_3$), *p*H 9.6. The sensitivities of the fast and slow ISEM methods were tested using 10-fold dilutions of either individual or group preparations diluted in 60 mM phosphate buffer, *p*H 7. Grids were examined using a JOEL 100C transmission electron microscope. Micrographs of five random fields on each grid were taken at a screen magnification of 33,000X. The number of virus particles within five random areas (each equivalent to 0.5 μm^2) of a contact print of each micrograph were counted, and the means and standard deviations were calculated.

"Decoration" was done to determine whether specific results could be obtained when using extracts containing mixtures of honeybee viruses. Mixtures containing particles of SBV and KBV, SBV and BQCV, SBV and CBPV, KBV and BQCV, KBV and CBPV, and BQCV and CBPV were each trapped on five EM grids by the fast ISEM method. Before negative staining, grids with each of the six mixtures, together with suitable controls, were floated for 15 min on 50-μl drops of rabbit antisera prepared against BQCV, CBPV, KBV, or SBV, or a hen antiserum against SBV.

A modification of the RIA method described by Kemp and Cowman (1981) was used to test for CBPV, BQCV, KBV, and SBV particles in 10-fold diluted extracts of individual and group preparations. CNBr-activated paper disks (Clarke et al., 1979) were prepared and stored in a vacuum over dessicant at 4°C. Drops (5 μl) of 10-fold dilutions of extracts of individual or group preparations were electrophoresed through slab gels of 0.75% Tris–EDTA-buffered agarose, *p*H 7.0, at 75 V/cm^2 for 2 hr. CNBr-activated paper, wetted to dampness

with a "binding buffer" consisting of 0.1 M $NaHCO_3$ and 0.1% v/v Triton X-100, was laid on the gel, and onto this were placed 20 sheets of dry filter paper and a uniform weight was applied. After all the fluid in the gel had passed into the dry filter papers through the damp filter paper (approx. 1 hr), the CNBr-activated paper was sandwiched between two filter papers dampened with "binding buffer" and left in a humid Petri dish overnight at room temperature to allow covalent binding of virus particles. It was then washed in 2% glycine dissolved in a "wash buffer" (consisting of 50 mM Tris–HCl, *p*H 8.0, 0.5 M sodium chloride, 0.1% Triton X-100), and finally washed in buffer for 1.5 hr. The position of bound virus particles was detected by incubating the paper in specific antiserum (0.235 ml/cm^2; reciprocal titer of each serum used is that listed for gel-diffusion tests in Table 1) for 1.5 hr, followed by six 15-min washes in "wash buffer." Finally, it was treated with ^{125}I-labeled protein A (Amersham Aust) for 1 hr, and again washed six times each for 15 min. The treated paper was air-dried and autoradiographed at −70°C using preexposed X-ray film. To confirm that the spots detected by RIA correlated with the position of virus particles in the gel, troughs were cut in identical gels parallel to the direction of electrophoretic migration, and these were filled with specific antiserum. After 3 hr, precipitin lines indicated the location of antigens.

The IgG for ELISA and for conjugation with urease was purified by the method described by Clark and Adams (1977). A 20-ml mixture containing 1.0 ml specific rabbit antiserum, 9 ml distilled water, and 10 ml saturated, neutralized ammonium sulfate solution was prepared and left for 1 hr at room temperature, and centrifuged at 1000*g* for 30 min to collect IgG, which was then dissolved in 2 ml of half-strength phosphate-buffered saline (PBS), *p*H 7.4. This was dialyzed three times against half-strength PBS overnight. The concentration of IgG was measured spectrophotometrically ($\lambda_{280\ nm}$), adjusted to 1 mg/ml, and stored in silicone-treated glass tubes at −18°C. A 5-ml (1 mg/ml) volume of partially purified IgG against SBV particles was conjugated with urease by the Commonwealth Serum Laboratories (CSL), Melbourne. Urease conjugated with protein A and with sheep anti-rabbit IgG were purchased from CSL. All conjugates were stored at −18°C.

To prepare F(ab′)$_2$ fragments, 0.25 ml of partially purified specific IgG (4 mg/ml) in 0.75 ml 0.07 M sodium acetate and 0.05 M sodium chloride was digested with 22.5 µl pepsin stock (1 mg/ml) by incubating overnight at 37°C (Adams and Barbara, 1982). Digestion products were removed by dialyzing against three changes of PBS buffer, *p*H 7.4.

The direct and indirect ELISA techniques were done in polystyrene microtiter trays (Dynatech M129B) as described by Koenig and Paul (1982). Tests were done to determine the optimum conditions using a checkerboard arrangement, dilutions of specific virus particles, and 10, 1.0, or 0.1 µg/ml dilutions of coating or probe serum, and 1:300, 1:200, or 1:100 dilutions of urease conjugated with protein A or with sheep anti-rabbit IgG immunoglobulins. After the optimum concentrations of reactants were estimated, the sensitivities of each method were determined using 10-fold serial dilutions of individual and group preparations. The sensitivity of the direct ELISA method was tested using dilutions of SBV particles, whereas the sensitivity of the indirect ELISA method was tested using dilutions of SBV, BQCV, KBV, and CBPV particles. The direct ELISA method was as follows: (1) Wells were coated with 200-µl aliquots of semipurified rabbit anti-SBV IgG (1.0 µg/ml) or their F(ab′)$_2$ fragments (5 µg/ml), diluted in 0.05 M sodium carbonate, *p*H 9.6, by incubating at 37°C for 2 hr. (2) Trays were rinsed three times by flooding wells with PBS, *p*H 7.4, containing 0.05% Tween-20 (PBS-T). (3) Al-

iquots (200 μl) of test sample in PBS-T containing 2% polyvinylpropylene (PVP) were added to duplicate wells and left at 4°C overnight. (4) Trays were rinsed three times with PBS-T. (5) Aliquots (200 μl) of urease-labeled rabbit anti-SBV IgG diluted 1:200 in PBS-T containing 2% PVP + 0.2% egg albumin were added to wells and incubated at 37°C for 3 hr. (6) Trays were rinsed three times in PBS-T, followed by three washes using distilled water. (7) Aliquots (200 μl) of undiluted urease substrate (CSL) were added to each well and incubated for 0.5 hr at 37°C, after which time the reaction was stopped by adding 10 μl of a 1% (w/v) aqueous (unbuffered) solution of "merthiolate" to each well.

The indirect ELISA method was as follows: (1) Steps 1–4 of the direct ELISA method were done, except that specific rabbit anti-virus $F(ab')_2$ globulin fragments (5 μg/ml) were used to coat wells. (2) Aliquots (200 μl) of specific rabbit anti-virus IgG diluted to 1.0 μg/ml in PBS-T containing 2% PVP and 0.2% egg albumin were added to each well, and then incubated for 3 hr at 37°C. (3) Steps 5–8 were done as described in the direct ELISA method.

RESULTS

Particles of BQCV, CBPV, KBV, and SBV in individual and group preparations produced precipitin lines against specific antiserum in gel diffusion tests and virus particles were seen in these preparations when they were examined by negative staining in the EM. Identical results were obtained when these suspensions were further concentrated in a microcentrifuge. Particles present in individual preparations could be detected by gel diffusion (using specific antiserum) and by negative staining in 10^{-1} and occasionally 10^{-2} dilutions, and in 10^{-2} and occasionally 10^{-3} dilutions of group preparations. However, some preparations, which initially gave no positive results in gel diffusion and negative staining, did so after further concentration. For example, when testing 10-fold dilutions of individual preparations by gel diffusion and negative staining, only some diluted to 10^{-1} gave positive results, whereas after being concentrated by microcentrifugation, some diluted to 10^{-2} were positive.

The bands of precipitate produced in gel diffusion tests using either rabbit or hen antiserum were confluent (Fig. 1).

The IO method detected BQCV, CBPV, KBV, and SBV particles in 10-fold dilutions of individual and group preparations diluted to 10^{-3} and 10^{-4}, respectively, when using specific antisera (Fig. 2, Table 2).

Both the fast and slow ISEM methods were used successfully to detect virus particles in individual and group preparations. However, there were statistically significant differences ($P < 0.05$) in the mean numbers of virus particles trapped after certain treatments: (1) More virus particles were trapped on EM grids treated with group preparations than on those treated with individual preparations (Figs. 3, 4). (2) The slow ISEM method trapped more virus particles on grids than the fast ISEM method (Figs. 3, 4). For example, the slow ISEM method detected SBV particles in individual and group preparations diluted to 10^{-3} and 10^{-4} respectively, but not when diluted more, whereas the fast ISEM method only detected SBV preparations 10 times more concentrated (Fig. 4). (3) Undiluted antisera were most efficient at trapping virus particles on EM grids using the fast ISEM method, whereas the most dilute antisera tested (1:1024) was most efficient using the slow ISEM method (Fig. 3). (4) More virus particles were trapped on EM grids treated with a rabbit serum than on grids treated similarly with a hen antiserum (Fig. 4). (5) SBV antiserum prepared in hens did not trap SBV particles on EM grids when used in the fast ISEM method. To check that this result was because hen antibodies did not react with protein A, other experiments were done using an antiserum against hen IgG, prepared in a rabbit by the methods described above. EM grids coated with protein A were floated on rabbit anti-

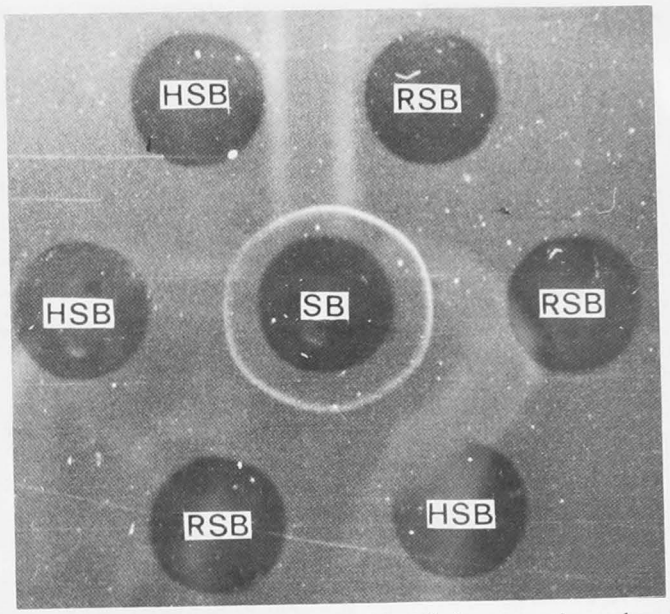

FIG. 1. Confluent bands of precipitate produced in gel-diffusion tests using sacbrood virus particles (SB) and specific antiserum, produced in a rabbit (RSB) and in a hen (HSB).

hen IgG, then on hen anti-SBV IgG, and finally on a SBV particle preparation, but few virus particles were seen. However, when a grid coated with protein A and then rabbit anti-hen IgG was floated on a SBV particle preparation incubated with anti-SBV hen serum, particles could be seen, but they were clumped and "decorated".

The "decoration" technique clearly resolves mixtures of virus particles, as particles were only "decorated" by homologous antiserum (Fig. 5). The most clearly

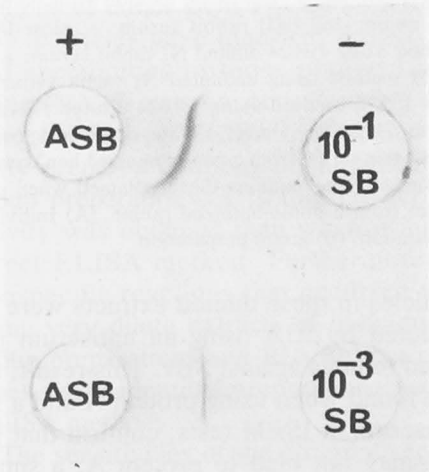

FIG. 2. Precipitin line formation in immunoosmoelectrophoresis tests using 10-fold dilutions of sacbrood virus particles (SB) and specific antiserum (ASB).

TABLE 2

AVERAGE DILUTION ENDPOINTS OBTAINED USING DIFFERENT SEROLOGICAL METHODS AND SPECIFIC RABBIT ANTISERUM TO ASSESS THE PARTICLE CONCENTRATION OF PREPARATIONS FROM INDIVIDUAL INFECTED BEES

Serological method	Dilution endpoint ($-\log_{10}$)			
	BQCV	CBPV	KBV	SBV
Gel diffusion	1–2	1–2	1–2	1–2
Immunoosmo-electrophoresis	3	2–3	3	3
"Fast" ISEM	NT[a]	1–2	NT	2–3
"Slow" ISEM	NT	1–2	NT	3–4
Radioimmunoassay	6–7	5–6	6–7	6–7
"Indirect" ELISA	6–7	5–6	6–7	6–7
"Direct" ELISA	NT	NT	NT	6

Note. The dilution endpoints obtained using extracts from 30 infected bees (group preparations) were on average 10 times greater than those obtained using individual infected bees. Virus acronyms are shown in Table 1.

[a] NT, not determined.

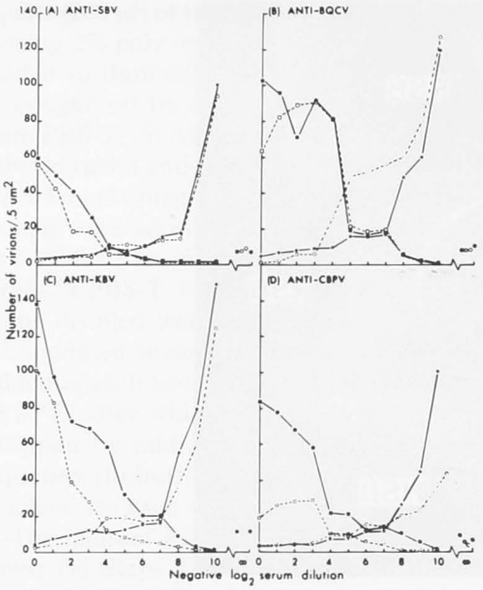

FIG. 3. The mean number of virus particles trapped on EM grids by the ISEM method using homologous antisera at different concentrations and undiluted virus preparations. The antisera were produced in rabbits, and are those described in Table 1. The virus preparations were from either 30 diseased individuals (group preparation), or from single diseased individuals (individual preparation). The "fast" and "slow" ISEM methods were used, and grids were treated as follows: □, Fast ISEM method and individual preparation; ■ fast ISEM method and group preparation; ○ slow ISEM method and individual preparation; ●, slow ISEM method and group preparation. The mean number of virus particles on control prepared grids are shown above the infinity signs.

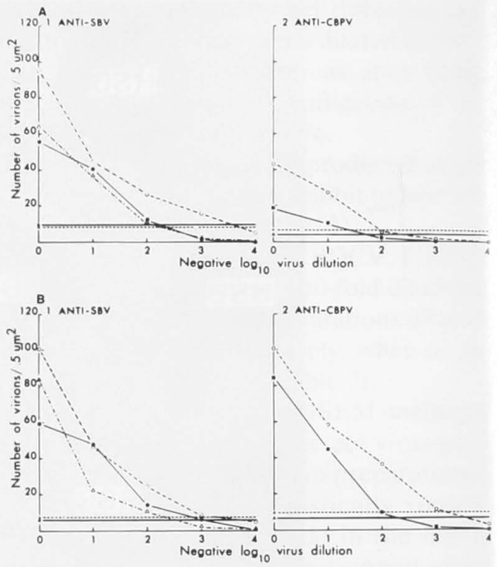

FIG. 4. The mean number of virus particles trapped on EM grids by the ISEM method using constant concentrations of antisera and differing concentrations of virus preparations. The antisera used were those described in Table 1; the virus preparations used were those described in Fig. 3. The "fast" and "slow" ISEM methods were used and grids were treated as follows: ○, slow ISEM method and rabbit anti-SBV serum; ●, fast ISEM method and rabbit anti-SBV serum; △, slow ISEM method and then hen anti-SBV serum; □, slow ISEM method and rabbit anti-CBPV serum; ■, fast ISEM method and rabbit anti-CBPV serum. The mean number of virus particles on control prepared grids are indicated as being smaller than the level shown by: ×, fast ISEM method using undiluted non-immunized (NI) rabbit serum; ∝, slow ISEM method using 1:1024 diluted NI rabbit serum; ∗, fast ISEM method using undiluted NI rabbit serum; ▲, slow ISEM method using 1:1024 diluted NI rabbit serum. The mean number of virus particles on control grids using serum from a nonimmunized hen were approximately the same as those obtained when using serum from a nonimmunized rabbit. (A) Individual preparation; (B) group preparation.

decorated particles were those that were treated with undiluted antisera. The reciprocals of titers obtained in the decoration tests are shown in Table 1. Hen SBV antiserum-decorated SBV particles better than rabbit SBV antiserum.

The RIA and ELISA methods were by far the most sensitive of the methods used for detecting virus particles in individual and group preparations.

Using RIA and individual and group preparations, BQCV, KBV, and SBV particles were detected (using specific antiserum) in extracts diluted to 10^{-6} and 10^{-7} respectively, and CBPV particles were detected in these extracts diluted to 10^{-5} and 10^{-6}, respectively. However, SBV particles in these diluted extracts were not detected by RIA using an antiserum prepared in hens against SBV. This result, and that found when using protein A and a hen antiserum in ISEM tests, confirm that hen IgG may not bind to protein A, a similar phenomenon found with rat IgG (Goding, 1980). Nonspecific reactions occurred when using RIA tests and very dilute extracts, making detection of dilution end-

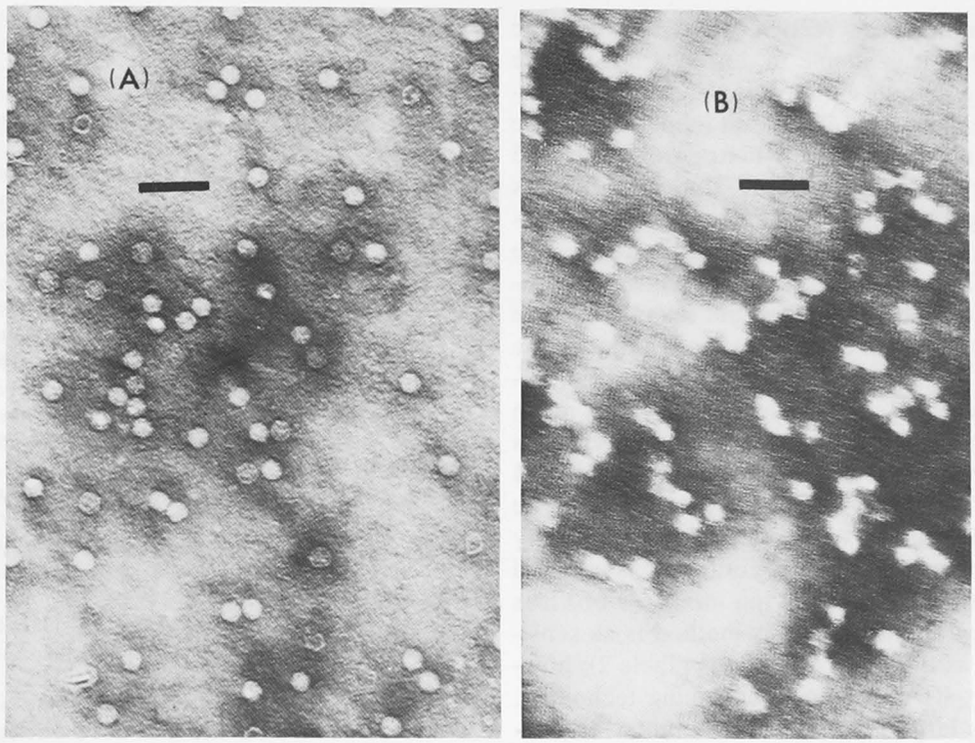

FIG. 5. Electron micrograph of (A) sacbrood virus (SBV) particles and (B) SBV particles "decorated" with specific rabbit antiserum. Bar = 100 nm.

points of virus suspensions difficult to assess unequivocally.

Direct and indirect ELISA tests using 10-fold dilutions of individual and group preparations of either BQCV, CBPV, KBV, or SBV particles gave identical sensitivities and specificities to those obtained when using RIA. However, occasionally, when using the indirect ELISA method and similarly prepared dilutions of individual and group preparations, a 10-fold greater sensitivity was obtained than when using the direct ELISA method. Furthermore, the nonspecific reactions that occurred when using very dilute extracts of individual or group preparations and RIA did not occur when using identical preparations and the ELISA methods.

The sensitivities of the different serological methods are summarised in Table 2.

DISCUSSION

The new fast and sensitive serological methods for detecting viruses seem not to have been used for detecting virus particles in extracts of diseased honeybees and, even though they have been used to assay virions of other insect viruses (Ohba et al., 1977; Crawford et al., 1978; Kelley et al., 1978; Crook and Payne, 1980; Longworth and Carey, 1980; Langridge et al., 1981; Scotti and Wigley, 1982), there are few reports comparing their effectiveness or their advantages and disadvantages for insect virology.

My results indicated that ISEM and IO were about one thousand times more sensitive than gel diffusion and negative staining in detecting virus particles in extracts from diseased bees. RIA and ELISA were about one million times more sensitive than gel diffusion (Table 2). However, even though each of the methods tested was effective in detecting and identifying virus particles, each had distinct advantages and disadvantages. For example, the gel-diffu-

sion method is relatively insensitive (Table 2), but it is simple to do, does not require large amounts of reactants, uses inexpensive materials, and is fast (about 4 hr). The IO method is even faster (about 15–45 min), more sensitive (Table 2), and uses very small amounts of reactants, but requires electrophoresis equipment. The ISEM method was about the same sensitivity as the IO method but requires the use of an electron microscope and, although the fast ISEM method takes as long as gel diffusion, the slow ISEM method takes much longer. However, the slow ISEM method is slightly more sensitive than the fast ISEM method. The ISEM methods would be rather tedious if used to examine many preparations, but "decoration" is a useful technique for resolving mixtures of virus preparations. The RIA method is as sensitive as the ELISA methods (Table 2); however, RIA is a time-consuming technique, uses large amounts of reactants, and requires the use of expensive and hazardous materials. Furthermore, nonspecific reactions occur when using RIA and very dilute test extracts, and this limits its effectiveness. On the other hand, this problem is not encountered using ELISA and, furthermore, ELISA does not use large amounts of reactants, the materials used in the tests are safe, durable, and relatively inexpensive, and many samples can be examined in the test. However, ELISA requires lengthy reaction times, though this is minimized by the direct ELISA method without much loss of sensitivity (Table 2).

The simple materials required to do gel diffusion and ISEM tests makes them suitable for field diagnostic work, but the ELISA technique would be more suitable than these if the test reaction times could be reduced, as the method has the advantages described above. IO, RIA, and ELISA are very useful laboratory techniques; the latter may be useful for determining the presence of viruses in organs, e.g., salivary glands, of individual bees.

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