A study of sacbrood and Kashmir virus infection in pupae of the honey bee, Apis mellifera

by

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

December, 1985

Frontispiece

(A) The Waite Agricultural Research Institute experimental apiary in late winter.

(B) Some developmental stages of Apis mellifera. L to R shows a final-instar larva, a pre-pupa, then four pupae at increasingly advanced stages of development. A 'white-eyed' pupa (see text Section 4.2.3) is shown third from left, a 'brown-eyed' pupa is fifth from left. Bar = 1 cm.


A


B

DECLARATION

I declare that the material contained in this thesis is my own work, unless otherwise acknowledged, and that it has not previously been submitted for consideration as part of any academic award. I consent to photocopying of the thesis, conditional on award of the degree.

ACKNOWLEDGEMENTS

I would like to thank the following people for the help which they have given to me:

: For advice throughout the project, my supervisors, Prof. D.E. Pinnock and Dr. R.I.B. Francki

: For assistance with statistical analyses, Ms. M. Morris and Ms. S. Waite

: For gifts of antisera, Dr. L. Bailey (Rothamsted Experimental Station, U.K.)

: For assistance with field collection of honey bees, Dr. M. Hornitzky and Mssrs. R. Winn, D. Brown and F. Lacey (N.S.W. and S.A. Departments of Agriculture) and for loan of Sminthopsis, Dr. M. Smith (S.A. Museum)

: For access to, or assistance with, various facilities or processes, Drs. R.I. Sommerville and M.C. Geddes, Mssrs. R. Miles and B. Palk, and staff of the Diagnostic Virology Unit at the Institute of Medical and Veterinary Science, Adelaide.

: For friendship and support, the staff and students of the Entomology Department, and in particular the staff of the Insect Pathology Lab, and my friends Andy and Paul.

: For assistance with proof-reading, and for her understanding throughout the project, my wife, Karen Gibb

I gratefully acknowledge the financial support provided by the University of Adelaide through a University Research Grant.

SUMMARY

Sacbrood and Kashmir bee viruses (SBV and KBV) are pathogens of the honey bee, Apis mellifera L. This thesis presents a study of the multiplication of these viruses in honey bee pupae, and describes some of the physiological and histopathological consequences of the infection of this host.

Each virus was studied as three types of infection - as laboratory initiated single and mixed (SBV with KBV) infections, and as inapparent infections in seemingly healthy field-collected pupae.

Laboratory studies showed that KBV was about one hundred times more infective than SBV, as measured by the minimum infective pupal dose, but at completion of multiplication similar amounts of each virus (about 300 µg) were recovered from each inoculated pupa. However, quantification of yields per pupa following inoculation with serial dilutions of SBV or KBV revealed significant variations in the extent of multiplication of each virus.

KBV was found to replicate in pupal haemocytes and oenocytes, and in cells of the epidermis, tracheal epithelium, muscle, foregut and hindgut. With the exception of oenocytes, replication of SBV was noted in the same locations, and was also observed in glands of the alimentary system, and in glial cells of the nervous system.

The multiplication of SBV in pupae was without detectable pathological effect, but KBV infection was found to cause rapid physiological perturbation and severe histological damage, culminating

in death of the host some 48 hr post-infection. In addition to causing direct damage to infected cells, KBV infection was associated with indirect damage to chiasmata cells in the optic lobe and to glial cells in other parts of the nervous system. Such damage was possibly caused by an unidentified, low molecular weight substance(s), perhaps present within healthy cells, and presumed to be released into the haemocoel by virally-induced lysis of infected cells.

The establishment and multiplication of SBV and KBV in mixed infections was found to be influenced by both the amount of each virus inoculated, and by the length of time separating the inoculations. When pupae were inoculated with both viruses, each at the minimum concentration required to initiate a single infection, it was found that mixed infections could become established only if SBV was allowed to multiply for at least five hours before inoculation of KBV. At these dose levels a shorter delay between the inoculations led to infection with KBV alone. However, even when inoculated 40 hours later than SBV, KBV rarely failed to infect pupae. Quantitative assays of each virus showed that progressively longer delays between inoculation of SBV and KBV increasingly favoured the multiplication of SBV, and reduced the multiplication of KBV. When the multiplication of each virus was approximately balanced, mixed infections could occasionally be recognised in individual cells of infected tissues. Damage to the nervous tissues of pupae with mixed infections was of a type similar to that observed in pupae infected with KBV alone.

Inapparent SBV and KBV infections were found in field-collected pupae during three consecutive summers. KBV infections could be activated by inoculation of pupae with small volumes of certain rabbit

sera, and in such cases produced virus yields similar to those recorded from overt infections. SBV was found in highly variable amounts, seemingly unrelated to the experimental treatment of pupae. In view of the consequences of overt infection by each virus, it is considered that the SBV infections should be described as inapparent, while such KBV infections may represent an example of true viral latency. Rigid controls suggested that the presence of inapparent infections did not influence the outcome of the experimental infections described earlier.

Addenda & Corrigenda

p6, 2.3, para 3; replace text starting line 4.

"The sample was divided into three equal groups, one of which was washed in the manner described above, while the other two remained unwashed. The washed group, and one of the unwashed groups, were then inoculated with sterile 2/3 strength Insect Ringers solution and incubated as described in Section 2.4. The second unwashed group was incubated without further treatment".

p7, 2.4, para 1; add the following sentence.

"Pupae were injected through an abdominal intersegmental membrane".

p8, 2.5, line 15 to read:

"...a major and a minor band".

p17, 3.3.1, para 1.

Note that homogenised pupae were washed as described in Section 2.3.

p21, 3.1, para 2; add the following:

"For the purpose of this thesis, latent and inapparent infections were both considered to be types of persistent infection. In general, the term 'latent' was used in the context suggested by Mims (1982), who defined latency as a "stage of persistent infection in which (a) microorganism causes no disease, but remains capable of activation and disease production". The term 'inapparent' was then used in cases where virus multiplication could be detected, but where symptoms of disease were not evident".

p33, footnote to 4.2.3.

Survival of pupae to metamorphosis was tested as part of an uncompleted (and hence unincluded) experiment. Pupae inoculated with either an lD100 of SBV or the same quantity of saline solution (2/3 strength Insect Ringers solution; 2/3 IR) were found to develop to metamorphosis at the same rate.

All pupae experienced emergence difficulties at metamorphosis, whether uninoculated, or inoculated with SBV, 2/3 1R, phosphate buffer or distilled water. In all cases this difficulty was apparently due to failure to completely emerge from the pupal exuvium, and was tentatively explained as resulting from the artificial culture conditions, ie, without the proximity of the walls of the brood chamber no mechanical or abrasive assistance was available to the emerging adult. These observations are based on a total of 317 pupae so challenged.

p86, 5.4.3:

Note that the term 'mock-infected' is used as synonym for '2/3 1R-inoculated'

p118, 7.2, lines 1 & 20

Underline Sminthopsis macroura and Mus musculus, respectively.

p124

Note that description of APV strains is contained in

Ball, B.V. (1985). Acute paralysis virus isolates from honeybee colonies infested with Varroa jacobsoni. Journal of Apicultural Research 24, 115-119.

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CHAPTER 1 : INTRODUCTION

The association between viruses and insects is a close and complex one, and entails important social and ecological consequences for a vast number of other living organisms.

The most important aspect of this relationship stems from the ability of vector insects to transmit the viruses responsible for diseases as diverse as human yellow fever and barley yellow dwarf. Another facet of this association, however, involves the interaction of insects with their own viral pathogens.

The International Committee on Taxonomy of Viruses (ICTV) recognises eleven families of viruses which have members capable of infecting insect hosts (Matthews, 1982). The Baculoviridae is one of the most thoroughly studied of these families, mostly because its members are both large in size and restricted to arthropods in their distribution. This latter fact makes these viruses of practical and economic importance for the control of insect pests.

In contrast, the 'small RNA viruses' of insects are probably the least well understood, with respect to both their taxonomic and their ecological relationships. The term 'small RNA virus', as used in general, and throughout this thesis, describes an isometric non-occluded virus of about 30 nm diameter which has a single stranded RNA genome. Viruses of this type have been found to infect members of all the major insect orders, and have also been isolated from many other invertebrates, as well as from vertebrates and plants.

For the last decade there has been substantial interest in the taxonomy of the small RNA viruses of invertebrates (see, for example, Longworth, 1978 and 1983 ; Moore and Tinsley, 1982) and there now appears to be general agreement on the framework required for a definitive scheme of classification.

It is generally accepted that three recognised virus families - the Picornaviridae, the Nodaviridae, and the as yet un-named family of Nudaurelia β - like viruses have members which infect insects (Matthews, 1982), and it seems probable that the Caliciviridae will also be added to this list (see Hillman et al., 1982). Because relatively few viruses can be assigned with confidence to these families, most schemes of classification also include a category of 'miscellaneous' or 'undefined' viruses. Although authors vary on the actual viruses included in this category, in all cases it is the largest group, showing the great amount of work which remains to be done in this field.

Small RNA virus infection is known to be of great ecological and economic importance to some communities of plants and vertebrates, but its significance for insect populations is less clear. Although the role of such viruses in epizootics of insect pests has been rarely documented (Reinganum et al., 1981), their detrimental effects in populations of beneficial insects, such as silkworms and honey bees, are recognised.

Viral diseases of honey bees have been probably better studied than those of any other species of insect. This situation results mainly from the economic importance of Apis mellifera in the pollination of field and orchard crops, and in the production of wax and honey. Husbandry of bee colonies by humans has provided ample opportunity for observation of

disease symptoms, and the economic importance of these colonies has encouraged research aimed at ameliorating the consequences of infection.

To date some 14 viruses have been described from honey bees (see Bailey, 1981), of which nine, including sacbrood and Kashmir viruses, are of the 'small RNA' type.

'Sacbrood' is a very common disease of bee larvae. Its viral aetiology was first suggested by White in 1917, and some fifty years later was confirmed by Bailey et al. (1964). Sacbrood virus (SBV) now has a world-wide distribution (Nixon, 1982), probably as a result of its simultaneous introduction with honey bee stocks. In Australia the virus is enzootic, although it occasionally reaches epizootic proportions and causes serious losses in apiaries. Infection with SBV usually causes death at the pre-pupal stage, but the virus can also replicate in adult workers without causing obvious disease (Bailey, 1981). Taxonomically, SBV has been tentatively assigned to the Picornaviridae (Moore and Tinsley, 1982), pending the necessary study of its replicative strategy (see Longworth, 1983).

In contrast to SBV, very little is known about Kashmir bee virus (KBV). This virus was originally included in Longworth's 'Group 5' (1978), but has since been relegated to an 'undefined' category by Moore and Tinsley (1982). KBV was first found in Apis cerana F. in northern and western India (Bailey and Woods, 1977 ; Bailey et al., 1979) where it was held responsible for losses of many bee colonies (Bhambure and Kshirsagar, 1978). The virus has also been detected in southern and eastern Australia, where a number of serological variants have been isolated from A. mellifera (Bailey et al., 1979). Although first

recognised in adult bees, it is now known that KBV can infect all stages of the life cycle (Hornitzky, 1981, 1982). Bee losses attributable to this virus are usually associated with localised epizootics, but neither the factors responsible for the onset of such outbreaks, nor the strategy of viral persistence have been clearly defined.

Given the limited nature of these and other reported results (as detailed in appropriate chapter introductions), it was clear that a number of aspects of the biology of both SBV and KBV warranted further study. The project described in this thesis was thus designed with three main aims, as follows :

(i) to make a comparative study of the multiplicative processes of SBV and KBV,

(ii) to investigate some of the physiological and histopathological consequences of such infections, and

(iii) to relate laboratory findings to published field observations, thus contributing to a more complete description of the ecology of each virus.

Because these aims were so diverse, each chapter of this thesis contains its own introduction and discussion sections, and in each of these the rationale and results of various experiments are placed in context with the relevant literature. The final chapter then contains a short overview of the entire project.

CHAPTER 2 : GENERAL MATERIALS AND METHODS

2.1 Introduction

The materials and methods used for experiments described in this thesis have been divided into two groups. Details of the first of these groups - basic procedures used throughout the project - are given in this chapter, but are not repeated in the chapters which follow. Details of those in the other group, i.e., the more specialised techniques used for only one facet of the work, are provided in the appropriate place. Subsequent mention of such a technique, if required, is by cross-reference to that section in which it was first described.

2.2 Bees

All experiments described in this thesis were done with worker caste pupae, a choice based on their ready availability, and ease of manipulation and maintenance. Furthermore, since later stage pupae may be regarded as pharate adults, their response(s) to infection might also be expected to reflect those shown by that part of the honey bee population.

Pupae were obtained from several sources. For most experiments pupae were from the Waite Agricultural Research Institute experimental apiary in Adelaide ($34^{o}56'S$, $138^{o}36'E$), but samples from the mid-north of South Australia (Rowland Flat, $34^{o}35'$, $138^{o}56'$; and Spalding, $33^{o}30'$, $138^{o}37'$), the south-east of South Australia (Mount Monster, $36^{o}07'$, $140^{o}20'$; Mundulla, $36^{o}22'$, $140^{o}42'$ and from near Naracoorte,

36°58', 140°45'), and from Glenfield, New South Wales (33°58', 150°54'), were also used in the study of inapparent infections.

2.3 Selection and preparation of pupae

After collection of brood frames, extracts of randomly selected pupae were tested for SBV and KBV by gel immunodiffusion (see Section 2.6), and checked by centrifugation on sucrose gradients to ensure that other viral infections were not present.

Before inoculation, pupae were washed twice in 4% formalin for 30 s and then rinsed twice in distilled water. The following experiment was done to demonstrate the efficacy of this method.

A sample of 45 pupae from a single hive was surface contaminated by immersion in 50 mM potassium phosphate, pH 7.0, (KP buffer), containing KBV at a concentration of 0.5 ng/ml. The pupae were drained, and then dried for 30 min at 34°C. The sample was divided into three equal groups, two of which were washed in the manner described above, while the third remained unwashed. The unwashed group, and one of the washed groups, were then inoculated with sterile 2/3 strength Insect Ringers solution (2/3 IR, full strength IR = 156 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$) and incubated as described in Section 2.4. The second washed group was incubated without further treatment.

After 48 hr, pupae from each treatment were individually homogenised in 200 µl of KP buffer, and tested for infection by double immunodiffusion. Ten of the unwashed, inoculated pupae were found to be infected with KBV, but no infection was detected in pupae of the washed,

inoculated group. One pupa in the unwashed, uninoculated group also became infected.

On the basis of these results it was concluded that the washing procedure was effective in preventing infection derived from surface contamintion by virus.

2.4 Inoculation

Inocula were prepared by dilution of concentrated preparations with sterile 2/3 IR, and were administered using a sterile disposable assembly consisting of a plastic syringe, an Acrodisc® (0.45 µm) bacteriological filter, and a 26 gauge needle. This assembly was mounted in an Isco® Model M Microapplicator, and the dose was calibrated by adjusting the delivered drop volume to the known capacity of a microcapillary tube.

After inoculation, pupae were incubated in a humid environment at 32-34°C, for a period determined by the experiment, then either processed immediately or stored at -80°C until use. No adults emerged from such pupae within the usual 1 to 7 day incubation periods.

2.5 Sources and purification of viruses

SBV was isolated from bee pupae taken from the Waite Agricultural Research Institute apiary, and KBV, of unknown origin, was from our laboratory stock. Both viruses were identified using antisera obtained from Dr. L. Bailey (Rothamsted Experimental Station, UK). Samples of the viruses later sent to Dr. Bailey were confirmed as the common strain of

SBV from Apis mellifera, and as a KBV strain serologically indistinguishable from the Queensland 2 strain (Bailey et al., 1979).

Both viruses were purified using slight modifications of the methods described by Bailey (1981). Four to six pupae were homogenised in 500 µl of KP buffer and 250 µl of diethyl ether, the homogenate was emulsified with 250 µl of carbon tetrachloride, and then centrifuged at 8,000 *g* for 2 min. The supernatant was collected and overlaid on 20-50% (w/w) phosphate (KP) buffered sucrose gradients. Following centrifugation in a Sorvall SV 80 rotor for 100 min at 19,000 rpm, the gradients were fractionated, and the UV(254 nm) - absorbing band was collected and dialysed exhaustively against KP buffer.

About 600 µg of sucrose gradient-purified virus was mixed with phosphate (KP) buffered CsCl (1.40 gm/ml initial density) and centrifuged for 25 h at 37,500 rpm in a Beckman SW60 Ti rotor. Fractionation showed that each virus sedimented into a major and minor band. Examination by electron microscopy showed that these bands consisted of intact and empty virus particles, respectively. Virus collected from the major band was dialysed exhaustively against KP buffer, concentrated, and stored at -80°C until required. Virus concentrations were determined by spectrophotometry, assuming that $E^{0.1\%}_{260} = 5.0$.

2.6 Serology

Isopycnically purified virus was fixed with 0.25% glutaraldehyde, on ice, for one half hour, then dialysed exhaustively against 2/3

strength phosphate buffered saline (2/3 PBS, full strength PBS = 137 mM NaCl, 2 mM KH_2PO_4, 16 mM Na_2HPO_4, 3 mM KCl, quoted as cation molarity). Rabbits were injected three times intramuscularly and subcutaneously; on each occasion the inoculum was about 150 µg of fixed virus mixed thoroughly with an equal volume of Freund's complete adjuvant. The second and third injections were given one week and four weeks after the first. Blood taken from a marginal ear vein about 40 days after the first injection was clotted and then centrifuged. The antisera had titres of 1/128 to 1/512 as measured by gel immunodiffusion tests.

Double immunodiffusion tests were done in 0.75% (w/v) Agarose A in KP buffer, with 0.2% disodium EDTA and 0.2% sodium azide. Plates were incubated at 35°C in a moist atmosphere for 24-36 h.

The double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used for both qualitative and quantitative determination of each virus. The method was essentially that of Clark and Adams (1977), with some modification. Immunoglobulins were precipitated from antisera with an equal volume of sterile, ice-cold, saturated ammonium sulphate. After centrifugation at 8,000 *g* for 5 min and dialysis against KP buffer, the immunoglobulin fraction was purified by chromatography on Sephacryl S300 gel. This globulin was used both for coating antibody (1.2 µg/ml in 50 mM carbonate coating buffer, pH 9.6), and for conjugation with bovine alkaline phosphatase (Sigma, Type VII-S : E.C. No. 3.1.3.1.).

Dynatech® microELISA plates were coated with antibody (200 µl/well), incubated for 6 h at 35°C, and washed three times with KP buffer containing 0.05% (v/v) Tween 80. Samples diluted serially in KP

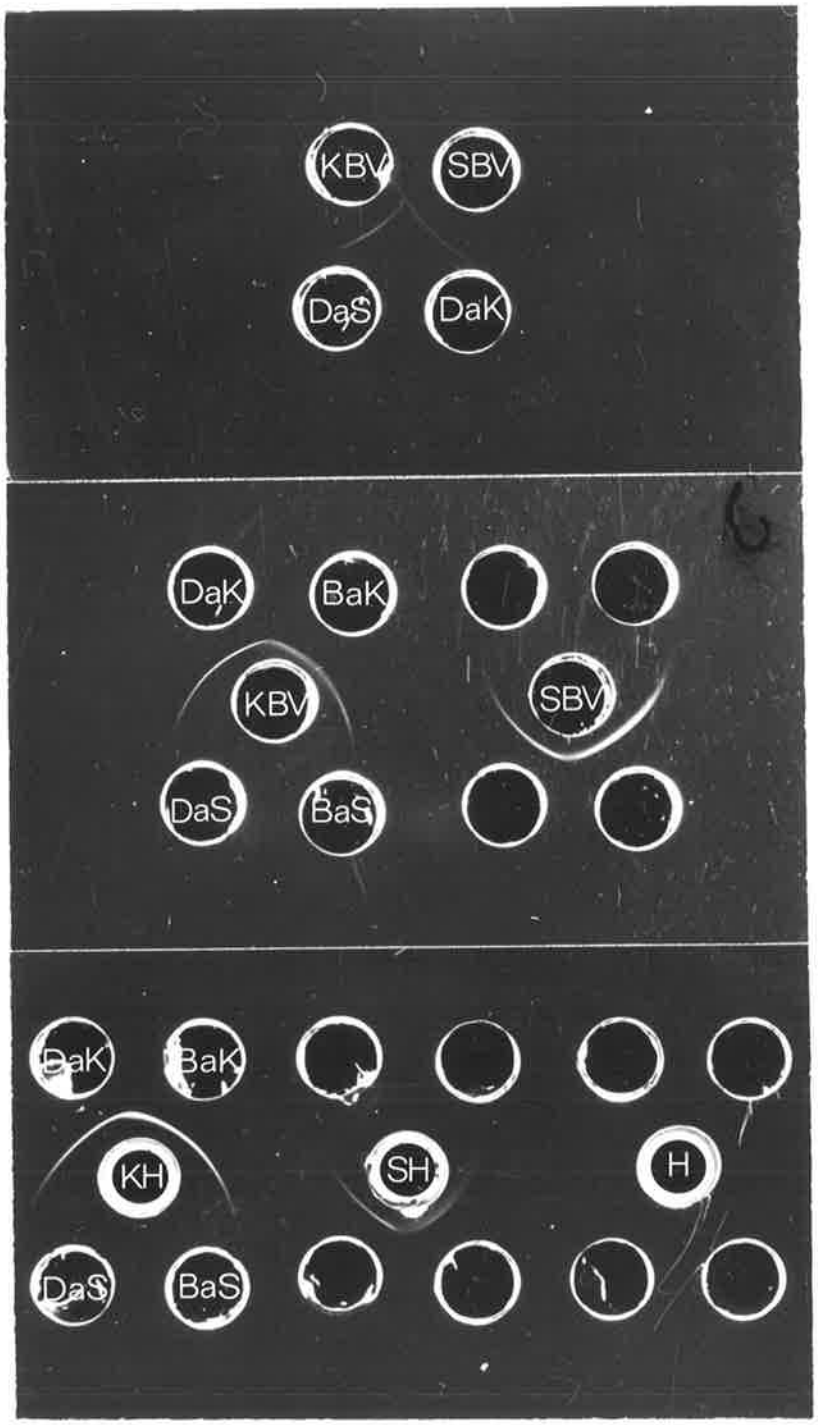
buffer with 2% (w/v) polyvinylpyrrolidone and 0.2% (w/v) ovalbumin were incubated in the plates overnight at 5°C. After washing the plates, conjugated antibody was added and incubated for 3 h at 35°C. After further washing, 200 µl of substrate buffer (9.7% (w/v) diethanolamine, adjusted to pH 9.8) containing 0.075% (w/v) p-nitrophenylphosphate was added to each well, and incubated at room temperature. After 30-45 min, absorbance at 410 nm was measured with a Dynatech® microELISA Minireader MR 590. Standard concentration curves were determined using purified virus. Less than 10 ng of each virus could be detected, but dilution of each sample for multiple testing gave an effective detection threshold of 100 ng of virus. No serological cross-reactivity was observed between KBV and SBV by this or the immunodiffusion technique (Figure 2.1), and host material did not interfere with the assays.

Figure 2.1

Determination of virus identity and antiserum specificity by double immunodiffusion. Contents of wells are as follows:

SBV, KBV	;	purified sample of each virus
SH, KH	;	virus with macerated infected host material
H	;	macerated healthy pupae
BaS, BaK	;	reference antisera to SBV and KBV, provided by Dr. L. Bailey
DaS, DaK	;	antisera to SBV and KBV prepared by author

Unlabelled wells contain antisera arranged in a sequence identical to labelled examples.


KBV
SBV
DaS
DaK
DaK
BaK
KBV
SBV
DaS
BaS
DaK
BaK
KH
SH
H
DaS
BaS

CHAPTER 3 : INAPPARENT INFECTION OF FIELD-COLLECTED PUPAE WITH SACBROOD AND KASHMIR VIRUSES

3.1 Introduction

While cases of acute and chronic viral infection are generally easy to detect and describe, problems often arise in the assessment of infections which present at a sub-clinical or 'inapparent' level.

This is especially true of infections of insects, where the situation is frequently exacerbated by limited knowledge of the ecology of the virus, and by poor choice of a descriptive terminology borrowed from plant and vertebrate virology. Thus, 'inapparent' infections are often referred to as being 'persistent' or 'latent' (see, for example, Evans and Harrap, 1982 ; Smith, 1976), even though the viruses responsible cause infections of unknown duration, and in most cases, are of unknown replicative status. The term 'inapparent infection' has been criticised by Moore and Tinsley (1982), as reflecting a lack of 'detailed and accurate field observations', but this very admission of that situation, coupled with the lack of implication about the status of infection, would seem to justify continued usage of the term.

There are numerous reports of inapparent viral infections of insects in the literature. One of the earliest is that by Bailey and Gibbs (1964), who showed that adult bees could harbour inapparent infections of acute paralysis virus (APV). Since that time, many other small RNA viruses have been found to cause similar types of infection, as discussed in Section 3.4. In addition, inapparent infections of cytoplasmic and nuclear polyhedrosis viruses are well documented (see,

for example, Jurkovicova, 1979 ; Tanada et al., 1964). In most cases such infections have been induced to multiply to detectable levels by introduction of foreign proteins into the host, either by contamination of food or by inoculation into the haemocoel.

In this study, two methods have been used to detect inapparent SBV and KBV infections in seemingly healthy pupae. The first was originally used in attempts to overcome cross-contamination during initial purification of these viruses, and involved inoculation of pupae with preparations of diluted rabbit antisera. Unexpected infection of these inoculated pupae demonstrated the presence of the viruses, and a second method, involving inoculation with homogenate prepared from sibling pupae, independently confirmed the validity of these results.

3.2 Detection of infections after inoculation of rabbit sera

3.2.1 Materials and methods

Inoculation of pupae with rabbit sera. Immune and pre-immune sera were collected from four rabbits and prepared as described in Section 2.6. Sera were diluted by 1/16 with sterile 2/3 IR for use as inocula. Each pupa received 3 µl of inoculum, was incubated for 4-5 days, and was then tested for infection by the ELISA method.

Analyses of rabbit sera. The total protein content of each serum was determined spectrophotometrically by the relation :

$$[\text{protein}]\ (\text{mg/ml}) = 1.5\ OD_{280} - 0.76\ OD_{260}\ \text{(Layne, 1957).}$$

Total IgG levels in the sera were determined by single radial immunodiffusion using dilutions of rabbit IgG (Sigma, I5006) as standards against goat anti-rabbit IgG antiserum (Sigma, R3128), the latter incorporated in 1% Agarose A in 0.85% NaCl. Osmolality of sera was measured in a Wescor Vapour Pressure Osmometer, Model 5100C, using verified Wescor standards.

3.2.2 Results

Tables 3.1 and 3.2 show the number of pupae in which infection with KBV or SBV, respectively, was detected, following inoculation with rabbit serum or 2/3 IR alone, or incubation without injection.

Activation of inapparent KBV infection was almost entirely confined to two treatments (Rabbit 1, anti-SBV; and Rabbit 3, pre-immune) while other treatments, in common with the uninjected controls, had little or no effect. Inapparent SBV infections were detected with similar frequencies in all treatments, including uninjected controls. For both viruses heterologous and pre-immune sera were more successful in activating inapparent infections than was homologous antiserum. This result is consistent with the usual neutralising ability of antisera directed against small RNA viruses (Matthews, 1982).

Each pupa was tested for the presence of both viruses, but in no instance was a mixed infection detected. The low incidence of infection of individual pupae, as shown by the data of Tables 3.1 and 3.2, prevented any demonstration of interaction between the viruses, even though the sample size was in excess of 1,600 pupae.

Table 3.1 : Activation of inapparent KBV infections in worker pupae of Apis mellifera by injection of immune and pre-immune sera.

Experiment No.	Date	Source of pupae	Hives tested	Rabbit 1 Anti-SBV	Rabbit 1 Pre-immune	Rabbit 2 Anti-SBV	Rabbit 2 Pre-immune	Rabbit 3 Anti-SBV	Rabbit 3 Pre-immune	Rabbit 4 Anti-KBV	Rabbit 4 Pre-immune	2/3 IR	Not injected
1	15.1.82	Waite*	3/3+	18/45#	1/45	-	-	-	-	0/45	-	0/45	0/45
2	3.2.82	Waite	3/3	19/45	4/45	-	-	-	-	0/45	-	1/45	1/45
3	11.3.82	Waite	0/1	0/30	0/15	-	-	-	-	0/15	-	0/15	0/15
4	10.1.83	Waite	1/1	0/18	0/14	0/18	0/12	0/13	4/19	0/18	0/14	0/15	-
5	14.2.83	Waite	2/2	0/23	-	-	-	0/23	2/23	0/23	-	-	0/23
6	24.2.83	mid-northern SA	0/5	-	-	0/60	0/60	-	-	-	-	-	0/60
7	1.4.83	south-eastern SA	0/6	0/60	0/60	-	-	-	-	-	-	-	0/60
8	11.1.84	Waite	0/3	0/30	0/30	-	-	0/30	0/30	-	-	0/30	-
9	23.1.84	Waite	0/2	0/24	0/24	-	-	0/24	0/24	0/24	0/24	0/24	-
10	8.2.84	Glenfield	1/1	2/15	0/15	0/15	0/15	0/15	1/15	0/15	0/15	0/15	0/15
Totals				39/290	5/248	0/93	0/87	0/105	7/111	0/185	0/53	1/189	1/263
Percentage infected				13.4	2.0	0.0	0.0	0.0	6.3	0.0	0.0	0.5	0.4

* Precise locations are given in Section 2.2.
\+ Numerator/denominator gives number of hives in which infection was detected/number of hives tested.
\# Numerator/denominator gives number of pupae in which infection was detected/number of pupae tested.

Table 3.2 : Detection of inapparent SBV infections in worker pupae of Apis mellifera following injection of immune and pre-immune sera.

		Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4			
Experiment No.	Hives tested	Anti-SBV	Pre-immune	Anti-SBV	Pre-immune	Anti-SBV	Pre-immune	Anti-KBV	Pre-immune	2/3 IR	Not injected
1*	3/3+	4/45#	1/45	-	-	-	-	13/45	-	13/45	4/45
2	2/3	0/45	1/45	-	-	-	-	1/45	-	0/45	0/45
3	0/1	0/30	0/15	-	-	-	-	0/15	-	0/15	0/15
4	1/1	0/18	0/14	1/18	2/12	0/13	1/19	0/18	0/14	0/15	-
5	1/2	0/23	-	-	-	0/23	1/23	0/23	-	-	0/23
6	0/5	-	-	0/60	0/60	-	-	-	-	-	0/60
7	3/6	0/60	2/60	-	-	-	-	-	-	-	3/60
8	2/3	0/30	6/30	-	-	1/30	0/30	-	-	1/30	-
9	0/2	0/24	0/24	-	-	0/24	0/24	0/24	0/24	0/24	-
10	1/1	1/15	1/15	2/15	2/15	0/15	1/15	2/15	1/15	2/15	0/15
Totals		5/290	11/248	3/93	4/87	1/105	3/111	16/185	1/53	16/189	7/263
Percentage infected		1.7	4.4	3.2	4.6	1.0	2.7	8.6	1.9	8.5	2.7

* For details see Table 3.1.
\+ # See footnotes to Table 3.1 for explanation

In Tables 3.1 and 3.2 the data have also been arranged to show the number of hives in which KBV and SBV, respectively, were found. Such treatment of data demonstrates that inapparent infections can be widespread within apiaries.

Quantitative determinations of virus growth were made on infected pupae from Experiment 1 (Tables 3.1 and 3.2) using the ELISA technique. KBV infections induced by serum inoculation produced 96 ± 8 µg of virus per pupa, while yields from SBV infections were highly variable, 304 ± 34 µg of virus being produced per pupa. For both viruses yields were similar to those produced in virus inoculation trials (see Section 4.2.2).

In an attempt to determine the serum component(s) responsible for KBV activation, measurements were made of the osmolality, and total protein and IgG concentration of each serum. The results of these measurements are shown in Table 3.3, but no common serum parameter which could be used to explain the results in Table 3.1 was found.

3.3 Detection of infections after inoculation of pupal homogenate

3.3.1 Materials and methods

Inoculation of pupae with pupal homogenate. Extracts of pupae were prepared essentially as described by Bailey et al. (1981). Twenty whole pupae were homogenised in 2 ml of KP buffer containing 0.2% sodium diethyldithiocarbamate (DIECA) and 1 ml of diethyl ether, and the homogenate was emulsified with 1 ml of carbon tetrachloride. The sample was centrifuged at 8,000 *g* for 7.5 min, and the supernatant was stored

Table 3.3 : Analyses of sera used as inocula for activation experiments.

	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4	
Serum parameter	Anti-SBV	Pre-immune	Anti-SBV	Pre-immune	Anti-SBV	Pre-immune	Anti-KBV	Pre-immune
Total protein (mg/ml)	99.4	94.0	90.0	85.0	76.2	77.2	98.4	91.0
IgG (mg/ml)	18.8	14.6	10.6	8.0	7.2	5.8	10.2	6.8
Osmolality (mmol/kg)	283	287	286	316	289	291	292	303

at 5°C for 24 hr. After repeating the centrifugation, 3 µl of supernatant was injected into pupae taken from the same hive as the homogenised sample. Pupae were incubated for 6 days, and held frozen until tested for infection by the ELISA method.

3.3.2 Results

Between December 1983 and February 1984, detection of inapparent infections by this method was attempted with 10 hives from the Waite Agricultural Research Institute apiary. Inapparent KBV infection was detected in pupae from only one of these 10 hives, and was apparently present at a low level, since only two of the 10 inoculated pupae from this hive became infected. Inapparent SBV infections were detected in pupae from four of the 10 hives, in a total of 19 of the 40 inoculated pupae from these hives.

3.4 Discussion

Before concluding that the detected overt infections resulted from activation of inapparent ones, the alternative possibility of contamination was examined. All the experiments reported in Section 3.2 were done with the same samples of sera, which were otherwise kept sealed. The many negative results, even with the 'active' sera, justified the exclusion of contamination, either of inocula or in any other way, as a likely explanation. The same negative results also precluded pupal response to the inoculation wound as an explanation, even though injury has been found to activate inapparent virus in another invertebrate system (Yudin and Clark, 1979).

Further evidence for the pupal origin of infection was provided by the homogenate inoculation experiments. Work presented in a later section (4.2.2) showed that when using purified virus, infection with SBV and KBV could be achieved by inoculation of as little as 10^{-5} or 10^{-8} ng/pupa, respectively. On this basis, inoculation of pupae with supernatant from a pupal homogenate was a sensitive test for infective virus in the homogenised sample, and although it was impossible to be certain that infection resulted from virus inoculation, rather than from activation, the pupal origin of the virus was indisputable.

As mentioned in Section 3.1, activation of inapparent infections has frequently been achieved by treatment of insect hosts with foreign proteins. In the most relevant example, Bailey and Gibbs (1964) used a variety of inocula including plant viruses, rabbit sera, and insect homogenates to induce inapparent infections of acute bee paralysis virus (APV) to multiply to detectable levels. Results from the present study show that not all rabbit sera appear to have the capacity to activate inapparent KBV infections, suggesting that, in this system at least, the presence of foreign protein in the haemolymph is not necessarily a sufficient stimulus for virus activation. Unfortunately, however, the measurements made did not reveal the identity of the stimulus.

In contrast to the response of KBV to different treatments, SBV infections were found in each experimental category, and also in non-injected control pupae. From this it seems likely that inapparent SBV infections occur commonly in pupae, and that such infections were detected, but not necessarily activated by the methods employed.

Given that both viruses may cause inapparent infections in pupae, a number of corollaries concerning the persistence and transmission of each virus can be suggested.

Thus, it is becoming increasingly evident that many small RNA viruses of insects may cause inapparent infections, as has now been documented for APV (Bailey and Gibbs, 1964), black queen cell virus (Bailey et al., 1981), cricket paralysis virus (Reinganum et al., 1970), KBV (this study) and SBV (Bailey et al., 1981, and this study). In this regard, these viruses resemble the human picornaviruses, which, in many cases, also cause sub-clinical infections. By definition, such infections cause little or no trauma to the host, but they have been shown to play a major role in viral persistence within human populations (see, for example, Fenner and White, 1976 ; Melnick, 1983). While the immune response of insect and vertebrate hosts differs, it seems probable that inapparent infections play a similarly important role in viral persistence within insect populations.

The results presented here contribute particularly to an understanding of the ecology of KBV in southern Australia. Bailey et al. (1979) and Bailey (1981) suggested that the virus might be enzootic in bees native to Australia and south-east Asia, but Anderson and Gibbs (1982) found no evidence of KBV, or of any other honey bee virus, in the native bee Trigona carbonaria Smith from eastern Australia. While the source of KBV infection of Apis mellifera in Australia remains unknown, the results presented here show that the virus can be maintained in honey bee populations without continual reinfection from a reservoir host species. Furthermore, fluctuations in the extent of inapparent infections, as detected in this study, provide a possible explanation

for the occasional KBV epizootics which occur in Australia (see Chapter 1). If these fluctuations are either caused or exacerbated by environmental factors (see Evans and Harrap, 1982) specific but unknown circumstances might well precipitate such outbreaks.

The biology of SBV has been studied extensively (see Bailey, 1981) and while the results presented here are in agreement with those in the literature, they also provide additional information.

Before this study, inapparent viral infections had been recorded only from adult bees (Bailey and Gibbs, 1964 ; Bailey et al., 1981), at which stage the source of infection is difficult to determine. The results reported here suggest that at least some of these infections may result from inapparent infection at the pupal, and hence at the larval, stage of development. Such larval infections are likely to be caused by contamination of food, although transovarial transmission cannot be discounted.

Transmission of SBV within the hive is thought to occur when newly emerged worker bees ingest virus particles, become infected, and then tend other healthy larvae (Bailey, 1969). While the hive cleaning behaviour of young bees predisposes them to ingestion of SBV, less than 1 µg of the virus multiplies in each adult worker (Bailey et al., 1981). Since some apparently healthy, uninoculated pupae were found to contain hundreds of µg of SBV, it is possible that inapparent infections provide an important source of transmissible virus. Adult bees emerging from such pupae may have the capacity to transmit SBV without further virus acquisition.

CHAPTER 4 : MULTIPLICATION OF SACBROOD AND KASHMIR VIRUSES

4.1 Introduction

In the broadest sense, viral multiplication can be studied at three different levels, using either whole animals, artificial cell cultures, or cell-free translation systems. While the choice between the alternatives is sometimes determined by practicability, the most important criterion is generally the purpose of the study. Thus, for example, in determinations of gross symptomology of infection, whole animal studies are likely to be the more useful, whereas for investigations of molecular events leading to virus synthesis the other two approaches are likely to be more informative.

Because no cultured bee cell line is available, considerations of both purpose and logistics dictated that experiments in this study should use field-collected, laboratory-infected pupae ; within this limitation investigations were done at the whole animal, cellular, and subcellular levels. On this basis the results have been split into the three sections which follow, although it should be realised that findings presented in each one must be interpreted in context with those in the other two.

The next few paragraphs provide a synopsis of the content, objectives, and literature pertaining to each of these sections.

Section 4.2 contains data from a series of preliminary experiments, designed to provide basic information about some important parameters of viral multiplication. In addition to their intrinsic interest, these

results gave some assurance that design of later experiments was appropriate for the intended purpose of the study. As discussed in 4.2.4, the results presented in Section 4.2 show close agreement with those of similar experiments summarised by Bailey (1976).

Three different methods were used to investigate the distribution of the viruses in pupal tissues, and are reported in Section 4.3. A number of authors have worked on the distribution of SBV in A. mellifera larvae and adults (Lee and Furgala, 1965a, 1967a & b ; Bailey, 1969 ; Mussen and Furgala, 1977) using infectivity assays and electron microscopy. In this study, acridine orange staining, fluorescent antibody staining, and electron microscopy were used to confirm and extend previously published results. Of these three methods, only electron microscopy proved suitable for investigation of KBV distribution, and the probable reasons for this are discussed. Since this is the first study of KBV replication at a cellular level, no direct comparisons can be drawn with other work, but apart from the absence of virus from tissues of the nervous system, the distribution was similar to that recorded for a number of other insect-infecting small RNA viruses.

In addition to mature particles, virus-infected cells generally contain a number of other structures not seen in healthy ones. This is true for cells infected with SBV and KBV, and Section 4.4 provides descriptions of the intracellular inclusions induced by these viruses.

While it has not been assumed that all virus-induced structures play an integral role in the viral replicative cycle, their study in other systems has often provided clues to the sub-cellular location(s)

and likely sequence of events leading to assembly of virus particles. The value of this approach has led to publication of extensive data, covering all the known groups of viruses (see, for example, Dalton and Haguenau, 1973 ; Maramorosch, 1977), however only viruses whose replication is similar in some respect to that of SBV or KBV have been mentioned in the discussion here (Section 4.4.3). As will be described, the replication of SBV has been found to be similar to that of picornaviruses (see Godman, 1973), but has been mostly compared with a specific example from the family - Theiler's murine encephalomyelitis virus (TMEV). Judged from structural evidence, the mode of KBV replication is quite dissimilar to that of SBV, and does not appear to closely resemble that of any other virus family.

4.2 Preliminary experiments

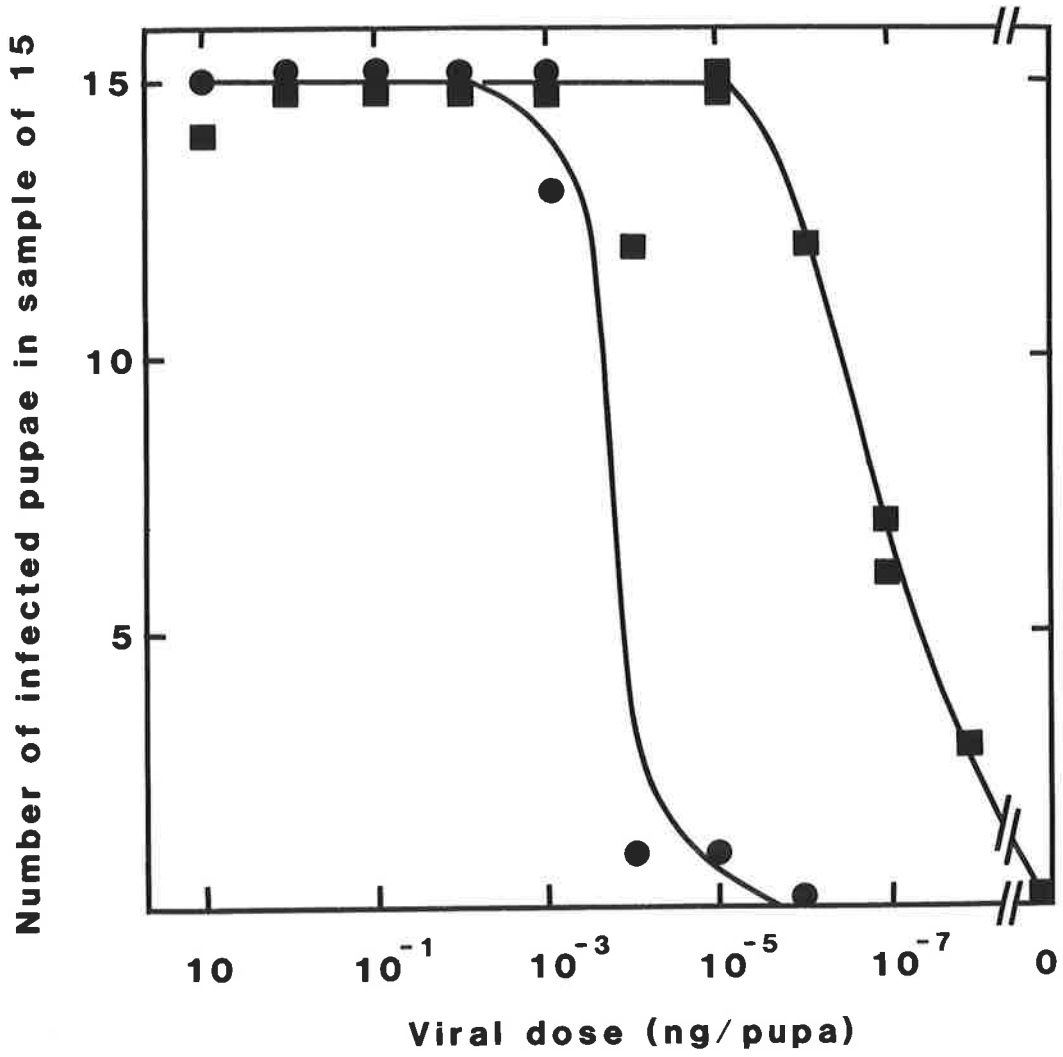
4.2.1 Viral infectivity

Figure 4.1 shows the infectivity of inocula containing different concentrations of virus. Pupae were taken from either two or three hives (SBV and KBV respectively; total sample size of 15 pupae/inoculum) and were each injected with a 3 µl inoculum containing between 10 and 10^{-8} ng of virus; after incubation for 7 days each was tested for infection using the ELISA method.

While the same hives were not used for both viruses, these results suggest that KBV was 10^2 to 10^3 times more infective than SBV when tested by inoculation. A dose of 10^{-8} ng/pupa was found to be sufficient to initiate KBV infection, while for SBV at least 10^{-5} ng/pupa were

Figure 4.1

Influence of inoculum concentration on establishment of viral infection in A. mellifera pupae. ● SBV ; ■ KBV.


Number of infected pupae in sample of 15
15
10
5
10
10^-1
10^-3
10^-5
10^-7
0
Viral dose (ng/pupa)

required. The dose with which every exposed pupa could be infected (ID_{100}) was taken to be 10^{-5} and 10^{-3} ng for KBV and SBV respectively.

4.2.2 Factors affecting viral multiplication

In addition to the qualitative assessment of viral infection described in Section 4.2.1, the same pupae were used in a quantitative study of viral multiplication, the results of which form the basis of Tables 4.1 and 4.2. For this study tests were made only on those pupae which had been exposed to an amount of virus equivalent to, or higher than, the ID_{100}.

Table 4.1 shows that infection by SBV resulted in the growth of some 200 to 300 µg of virus per pupa. Pupae from the different hives were found to differ in their response to varying inoculum concentration ('interaction' F-ratio = 6.59, $p < 0.001$) and this result precluded further statistical analysis of these data.

Similarly, Table 4.2 shows that infection by KBV produced 200 to 400 µg of virus per pupa; while there was no overall effect of inoculum concentration on virus production ('inoculum' F-ratio not significant), testing of results from individual hives revealed that for Hive 11 there was a highly significant relationship between the two variables.

Analysis of variance showed significant differences in the amounts of KBV produced in pupae from different hives ('hive' F-ratio = 3.13, $p = 0.05$), although the response across the range of KBV inocula did not vary from hive to hive ('interaction' F-ratio not significant). The overall means (with standard errors) were 298 ± 17, 345 ± 21

Table 4.1 : Quantities of SBV recovered from individually-tested pupae after inoculation with different amounts of virus, and incubation for 7 days.

	Inoculum (ng/pupa)				
Hive	10	1	10^{-1}	10^{-2}	10^{-3}
39	228 ± 20[#] (6)	226 ± 21 (8)	219 ± 19 (8)	221 ± 21 (8)	182 ± 13 (8)
48	197 ± 18 (6)	272 ± 13 (7)	232 ± 17 (7)	182 ± 9 (7)	304 ± 23 (7)

[#] Mean yield (µg) ± S.E. of mean, (number in sample). Bartlett's test : $X^2_9 = 3.87$ ($p > 0.05$)

	Line of best fit[#]	r	t-statistic (b = 0)	Sig
39	y = 211 + 1.85x	0.42	0.81	N.S.
48	y = 248 - 4.88x	-0.42	0.80	N.S.

[#] y = final quantity (µg) of SBV per pupa; x = quantity (ng) of SBV in inoculum.

ANOVA

Source	df	ss	ms	F	Sig
Hive ignoring inoculum	1	10469	10469	-[#]	
Inoculum eliminating hive	4	19831	4958	-	
Inoculum ignoring hive	4	19563	4891	-	
Hive eliminating inoculum	1	10737	10737	-	
Interaction	4	62697	15674	6.59	***
Error	62	147573	2380		
Total	71	240570			

[#] not applicable

Table 4.2 : Quantities of KBV recovered from individually-tested pupae after inoculation with different amounts of virus, and incubation for 7 days.

	Inoculum (ng/pupa)					
Hive	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-5}
11	440 ± 29[#] (4)	266 ± 31	270 ± 28	268 ± 36	298 ± 40	275 ± 39
33	442 ± 75	308 ± 32	368 ± 43	383 ± 46	282 ± 28	282 ± 43
42	341 ± 48	223 ± 23	397 ± 51	257 ± 26	282 ± 40	233 ± 18

[#] Mean yield (µg) ± S.E. of mean, (sample size of 5 unless shown otherwise). Bartlett's test : $X^2_{17} = 13.54$ ($p > 0.05$)

	Line of best fit[#]	r	t-statistic (b = 0)	Sig
11	y = 272 + 16.6x	0.97	8.11	**
33	y = 323 + 11.8x	0.73	2.18	N.S.
42	y = 278 + 5.8x	0.34	0.73	N.S.

[#] y = final quantity (µg) of KBV per pupa; x = quantity (ng) of KBV in inoculum.

ANOVA (mixed model)[#]

Source	df	ss	ms	F	Sig
Inoculum	5	242715	48543	1.97	N.S.
Hives	2	49394	24697	3.13	*
Interaction	10	108368	10837	1.38	N.S.
Error	71	559406	7879		
Total	88	959883			

[#] Computations include one calculated 'missing observation' for Hive 11, 10 ng/pupa inoculum.

and 289 ± 18 µg/pupa for hives 11, 33 and 42 respectively, and pairwise testing showed that the significant difference noted above was due to the high level of viral growth in pupae from hive 33, compared to that in pupae from the other two hives.

Analysis of fresh weights of pupae from these hives (Table 4.3) showed no significant difference between two age classes from the same hive, but described a highly significant difference between pupae from different hives (F-ratio = 16.55, $p < 0.001$). Comparison of the tabulated weights shows that in this case, too, significance resulted from the influence of pupae from hive 33, these being markedly smaller than pupae from the other two hives.

Results from a further experiment, in which pupae of these two age classes were infected with KBV, suggested that the age of the host had no significant influence on the extent of viral multiplication (Table 4.4).

4.2.3 Viral multiplication and host metabolism

The studies reported in 4.2.2 suggested that the extent of virus multiplication was not dependent on host age. It was, however, considered that pupae at various stages of development might be differentially susceptible to metabolic perturbations caused by viral multiplication, and that this in turn might influence the time of onset of pathological changes and/or pupal death. Since it was important not to confuse pathological and post-mortem tissue damage, an indicator of continued host metabolism, capable of reflecting the influence of viral multiplication, was sought. The development of pupal eye colour was

Table 4.3 : Fresh weights of two age classes of pupae from hives used in KBV growth experiment (Table 4.2).

Hive	White-eyed pupae (1)#	Brown-eyed pupae (7)
11	186 ± 3+ (4)	177 ± 3
33	160 ± 5	151 ± 5
42	179 ± 6	166 ± 5

Munsell eye colour score ; see Section 4.2.3

\+ Mean weight (mg) ± S.E. of mean, (sample size of 5 unless shown otherwise). Bartlett's test : $X^2_5 = 3.15$ ($p > 0.05$, N.S.)

ANOVA (mixed model)#

Source	df	ss	ms	F	Sig
Age	1	908	908	0.49	N.S.
Hives	2	3673	1837	16.55	***
Interaction	2	11	6	0.05	N.S.
Error	23	2561	111		
Total	28	7153			

Computations include one calculated 'missing observation' for Hive 11, white-eyed pupae.

Table 4.4 : Quantities of KBV recovered from individually-tested pupae of two different age groups, each inoculated with 10^{-5} ng of virus.

Age class	Virus recovered	t-statistic	Sig
White-eyed pupae (1)#	101 ± 12+	1.378	N.S.
Brown-eyed pupae (7)	111 ± 11		

Munsell eye colour score ; see Section 4.2.3

\+ Mean yield (µg) ± S.E. of mean, sample size of 10.

Test for equality of variances : $F_{9,9,0.025} = 1.4$ ($p > 0.05$)

found to be an easily observed character capable of fulfilling these requirements, and had the advantage that the use of invasive or destructive techniques were not necessary.

Table 4.5 shows the colours assigned to the increasingly pigmented eyes of a sample of pupae from a single hive, as they developed over a 72 hour period. Figure 4.2 shows the mean (n = 6) eye colour of control (2/3 IR inoculated) and infected pupae from two age classes of this sample, observed at 12 hourly intervals, and also reports the Kruskal-Wallis H statistic calculated from the ranked results of these observations.

From the data (Fig. 4.2) it appears that the two viruses have very different effects on host metabolism, as measured by eye colour development. SBV infection caused no detectable effect, but the eye colour development, and presumably metabolism, of pupae from both age classes was severely depressed by the presence of KBV. Figure 4.2 shows that this effect became apparent 36 hr post-infection in young pupae, but that a 60 hr post-infection period was required before it could be seen in the older pupae. For reasons discussed more fully in Section 4.2.4, this result cannot necessarily be interpreted as demonstrating the existence of age-dependent susceptibility to the virus. However, it may be concluded that in both age classes eye colour development ceased 48 hr after inoculation of KBV.

4.2.4 Discussion

The results presented in this section provide the first quantitative estimates of the infectivity of KBV, and of yields from

Table 4.5 : Pupal eye colour* assignations from Munsell Book of Colors (Munsell, 1929).

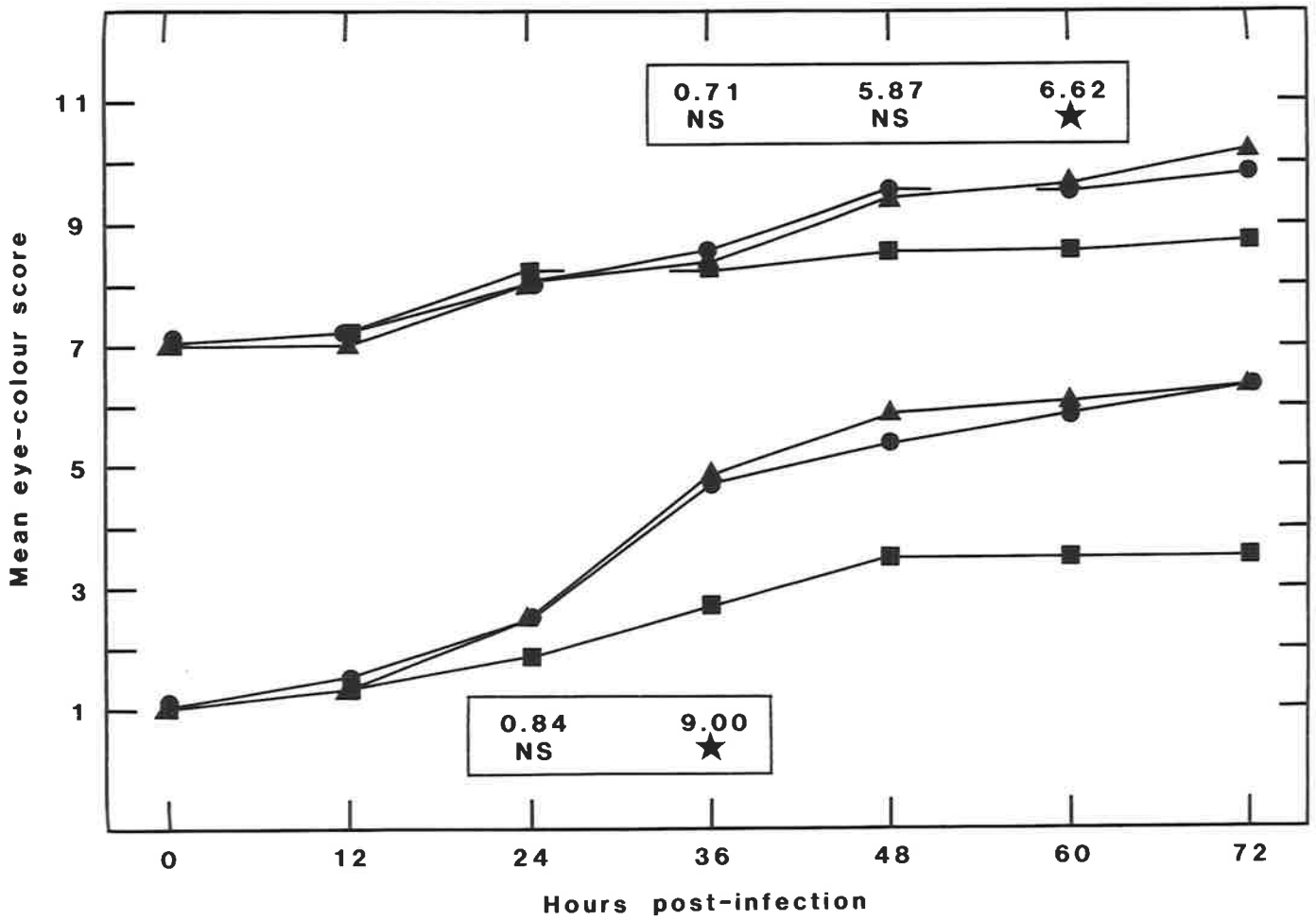
Eye-colour score	Munsell Colour Hue, value/chroma		Colloquial description
1	Yellow-red yellow,	7/4	White
2	Yellow-red,	6/4	
3	Red yellow-red (R YR),	6/4	Salmon Pink
4	R YR,	5/4	
5	R YR,	4/4	Light Brown
6	R YR,	3/6	
7	R YR,	3/4	
8	Red (R),	2/4	Chocolate
9	R,	2/2	Dark brown
10	Red-purple,	2/2	
11	Purple,	2/2	Purple-black

* Munsell reference colours and the pupae were simultaneously examined under standard lighting conditions, and the reference colour most closely corresponding to eye colour was recorded.

Figure 4.2

Development of eye colouration in healthy and virus-infected A. mellifera pupae. See Table 4.5 for details of 'eye-colour score' assignation, and Frontispiece for illustration. Insets show value and significance level of Kruskal-Wallis H-statistic.

▲ Control (2/3 IR) ; ● SBV ; ■ KBV.


0.71
NS
5.87
NS
6.62
★
0.84
NS
9.00
★
11
9
7
5
3
1
0
12
24
36
48
60
72
Mean eye-colour score
Hours post-infection

infected pupae of A. mellifera. The median infective dose (ID_{50}), estimated from Figure 4.1, was 5×10^{-7} ng/pupa (equivalent to about 35 virus particles/individual ; see Appendix A for calculations), and the mean yield was about 300 µg/pupa (about 2×10^{12} particles/pupa). These data indicate that KBV has an infectivity and productivity similar to that of acute paralysis virus (APV), which has been quoted by Bailey (1976) to have an ID_{50} of 10^2 particles by inoculation, and to produce some 10^{12} particles/individual. The data presented here thus partially support the assertions of Bailey (1982), who stated that KBV

> "multiplies profusely when injected into adult bees - or when simply rubbed on their bodies - and ... then kills them within two or three days".

In this study attempts to initiate KBV infection of adult bees by topical application of virus had variable success (data not shown). However, as reported, KBV proved both to replicate to high levels following inoculation into pupae, and to kill that host in about the same length of time (see below).

Results from Section 4.2.1 confirmed the relatively low infectivity of SBV for post-larval stages of the life cycle, with the ID_{50} of SBV determined in this study (about 5×10^{-4} ng; see Figure 4.1) being in close agreement with that of a previously published value (10^3 - 10^4 virus particles; Bailey, 1969). Both figures for SBV are much higher than estimates of the ID_{50} of APV (10^2 particles/individual, administered by inoculation; Bailey, 1976) and chronic paralysis virus (CPV; ID_{50} CPV quoted as 10^2 particles/individual by inoculation, Bailey, op.cit.). Yields of SBV reported in this study (200-300 µg/pupa) are in general agreement with several published estimates, viz., 100 µg

per adult drone head, 10^{13} particles (about 150 µg) per larva, and 'about a milligram' per larva (Bailey, op.cit. and 1981), but differ significantly from a report of 10^9 - 10^{10} particles (about 15-150 ng) per adult worker head (Bailey, 1969). The reason for this last discrepancy is not clear, but yields of 10^3 times that magnitude have consistently been recovered during this study.

In only one of the five infectivity trials was a significant relationship detected between inoculum size and final virus yield, leading to a decision that, whenever possible, future infections should be initiated with an ID_{100}.

Assessment of viral yields following tests with serially-diluted inocula revealed 'between' hive variation in pupal response to KBV infection, and while it is possible that such variation also existed for SBV, this could not be demonstrated, due to the significance of the interaction F-value (see Table 4.1).

Because the reported experiments were done almost simultaneously, and used brood from the same apiary, environmental or seasonal factors were unlikely to have been responsible for these differences. Rather, it seems more probable that the variation was due to genetically determined differences in susceptibility to infection, as has been demonstrated for several viruses in this and other insect hosts (for example, Rinderer et al., 1975 ; Briese, 1982). This variation in response showed that valid comparisons of virus yield could be made only between pupae from the same sample pool, and hence, that comparative quantitative studies should use pupae from only one hive.

In this work, assessment of host age and metabolic state was based on pupal eye-colour (see Frontispiece for illustration). Development of pigmentation in the insect eye is known to be due to formation of ommochromes and pterines via series of enzymically-mediated steps (see Chapman, 1971). On this basis, it was thought that objective assessment of the rate of eye-colour development might prove to be a satisfactory method for recognition of virally-induced perturbations of pupal metabolism. As described in Section 4.2.3, this was found to be so, but it was also found that, even in uninfected pupae, the rate of change was not constant throughout development ; as a consequence, the sensitivity of the technique was assumed to vary with pupal age. This meant that it was not possible to decide whether the rapid indication of KBV-induced metabolic disturbance in young pupae resulted from greater susceptibility of this stage (compared to older individuals), from an increased sensitivity of the test, or from elements of both. With this proviso, however, the technique fulfilled the requirements outlined in Section 4.2.3, and suggested a limit for the duration of KBV infection (48 hr) within which examination of viral pathology could be made.

As shown in Figure 4.2, 'brown-eyed' pupae were some 3 days older than 'white-eyed' pupae ; although this age difference was shown to have no effect on virus replication (Table 4.4), it was decided to standardise experiments to use white-eyed pupae (unless stated otherwise) because of the greater ease with which a uniform sample could be collected.

In summary, the aim of this section was to determine the influence of a number of variables on viral multiplication. The design of

experiments described later in this thesis make use of the results of the experiments presented here.

4.3 Sites of virus multiplication

4.3.1 Materials and methods

Fluorescent antibody staining. Antisera used for direct immunofluorescent staining were prepared as described (Section 2.6).

The method employed for conjugation of immunoglobulins with fluorescein isothiocyanate (FITC, BDH Chemicals Ltd.) was essentially that of Emmons and Riggs (1977). Globulins were precipitated from antisera with ice-cold 50% ammonium sulphate, concentrated by centrifugation, resuspended and dialysed against PBS, and quantified spectrophotometrically, assuming E_{280} 1.4 = 1 mg/ml. Sodium carbonate-bicarbonate (SC) buffer, 0.5 M, pH 9.0, was used to dissolve FITC, and to dilute the globulin solution to a final protein concentration of about 1%. FITC was added dropwise, with stirring, to buffered globulin solution in the ratio 1 mg dye to 100 mg protein, and the mixture was stirred overnight at 4°C. All steps involving FITC were done in subdued lighting conditions, and whenever possible, in darkness.

FITC conjugates were used without further purification, because specific staining activity was lost after either chromatography on Sephacryl S300 gel, or adsorption with acetone-dried powder prepared from healthy bee pupae (Nairn, 1969).

Numerous attempts were made to conjugate globulins with rhodamine B isothiocyanate (RITC, Sigma, mixed isomers) or crystalline tetramethylrhodamine isothiocyanate (TRITC, Nordic Immunological Labs., isomer R) using the method of Emmons and Riggs (1977) described above, and both the exact method, and variations, of that described by Amante et al. (1972). In no case was a conjugate with specific staining activity produced.

Before being used to stain frozen sections of pupae, conjugates were titrated against smear preparations. The same method was used to stain both sections and smears, and control specimens from uninfected pupae were processed in parallel with every experimental preparation. Smears or frozen sections (8 µm thickness, cut on an Ames Microtome-Cryostat II) were air dried on gelatin-coated, formalin-fixed microscope slides (Nairn, 1969). Material was fixed in acetone (10 min at room temperature), again air dried, then stained for 25 min at 34°C with conjugate in KP buffer and 25% foetal calf serum (Commonwealth Serum Labs.). After staining, the preparations were washed for a total of 15 min in two changes of SC buffer, and mounted in 50% SC-buffered glycerol.

Specimens were examined by incident light illumination at wavelengths of 450-490 nm, using a Leitz Ploemopak fluorescence attachment on a Leitz Orthoplan microscope fitted with a Wild MPS 45 Photoautomat system.

Acridine Orange staining. The method of Spendlove (1967) was slightly modified for use in acridine orange (AO) staining of paraffin-embedded tissue. Pupae were dissected and processed for light microscopy

as described (Section 5.2.1), except that Carnoy's fluid was used for tissue fixation. After hydration, sections were equilibrated in McIlvaine's buffer, pH 3.8, for 15 min (McIlvaine's buffer = 0.1 M citric acid with 0.2 M Na_2HPO_4, in the ratio 6.5:3.5 respectively). Sections were stained for 2.5 min in 0.01% acridine orange, washed for a total of 30 min and then mounted for examination, all in McIlvaine's buffer. The specimens were examined and photographed using the fluorescence microscopy system described above.

Electron microscopy. Pupae were dissected in 2.5% glutaraldehyde and 40% IR in 0.025 M potassium phosphate buffer, pH 7.0 (1/2 KP), and fixed for 4 hr at room temperature. The tissue samples were washed for a total of 3 hr in two changes of 0.025 M potassium phosphate buffer, pH 8.0, and then incubated with pancreatic ribonuclease (Type III-A, Sigma R5125) at a concentration of 2 µg/ml, in the same buffer, for 16 hr at 25°C (Hatta and Francki, 1981). Samples were post-fixed for 1 to 2 hr in 1% osmium tetroxide in 1/2 KP buffer, rinsed for 1 hr in two changes of 1/2 KP buffer, and dehydrated over a 4 hr period in a graded acetone series. Samples were infiltrated in 50% and 75% Spurr's resin diluted with acetone (Spurr, 1969), and then in one change of 100% Spurr's resin, over a period of 40 hr. Specimens were transferred to fresh 100% Spurr's resin, and polymerised at 70°C for 24 hr.

Thin sections were cut with a diamond knife on an LKB 'Ultratome IV' ultramicrotome, then mounted on uncoated copper or nickel grids and double stained with uranyl acetate and lead citrate. The specimens were examined in a JEM 100 CX electron microscope using an accelerating voltage of 60 KeV.

The resistance of the two viruses to RNase digestion was tested by mixing sucrose gradient-purified virus, either fixed as described above, or unfixed, with 0.025M potassium phosphate, pH 8.0, containing 0.0, 0.2 or 2.0 µg/ml of RNase (final concentration). The mixture was incubated for 21 hr at 25°C, then centrifuged on sucrose gradients and fractionated as described (Section 2.5). The size and shape of viral peaks recovered from the gradients (Figure 4.3) showed that both viruses were resistant to these concentrations of the enzyme.

4.3.2 **Fluorescent antibody staining**

Titration of fluorescein-conjugated rabbit antibodies with pupal smear preparations showed that optimum working concentrations were obtained at 15 to 30-fold dilutions of the original stock. Such dilutions revealed areas of bright fluorescence when tested against preparations from pupae infected with the homologous virus, but failed to stain either smears from pupae infected with heterologous virus, or those from uninfected pupae. Examples of such staining reactions are shown in Figure 4.4(a) and (b).

Specific fluorescence reactions were also seen in sections of pupae prepared by cryomicrotomy and stained with conjugates ; such reactions provided some insight into SBV multiplication, but proved to be of little value for interpretation of KBV multiplication.

Examination of sections from pupae frozen 20 hr after SBV inoculation revealed only very low levels of fluorescence. The great majority of such fluorescence was in haemocytes [Figure 4.5 (a) and (b)], though there were also faint indications of infection in the

Figure 4.3

UV (254 nm)-absorbing peaks recovered from sucrose gradients following centrifugation of virus incubated with RNase. Series a-c and g-j show unfixed SBV and KBV respectively, after incubation with RNase at concentrations of 0.0 (a and g), 0.2 (b and h) or 2.0 µg/ml (c and j). Series d-f and k-m show glutaraldehyde-fixed SBV and KBV respectively, following incubation with RNase using the concentrations and sequence described above. See Section 4.3.1 for experimental details.

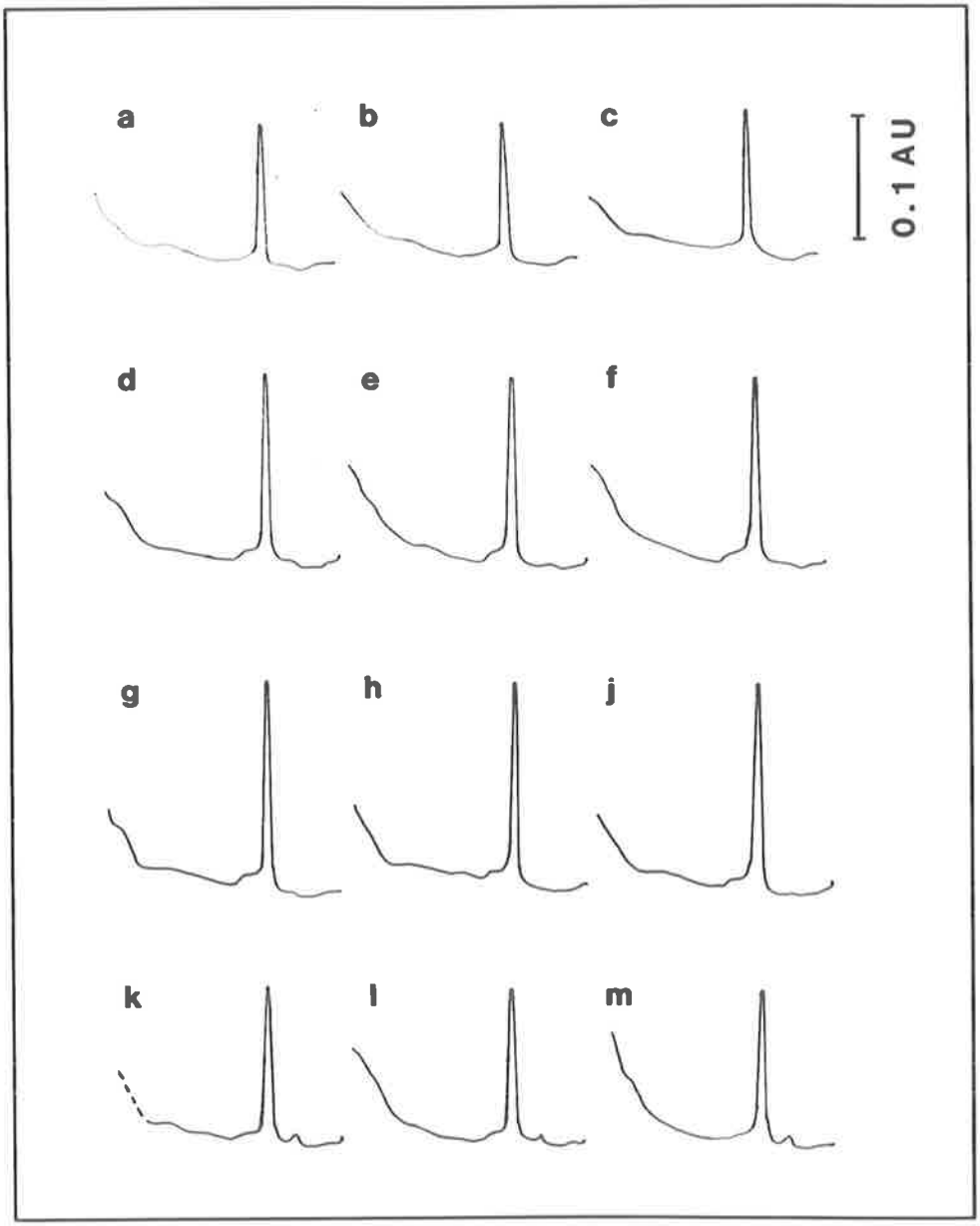
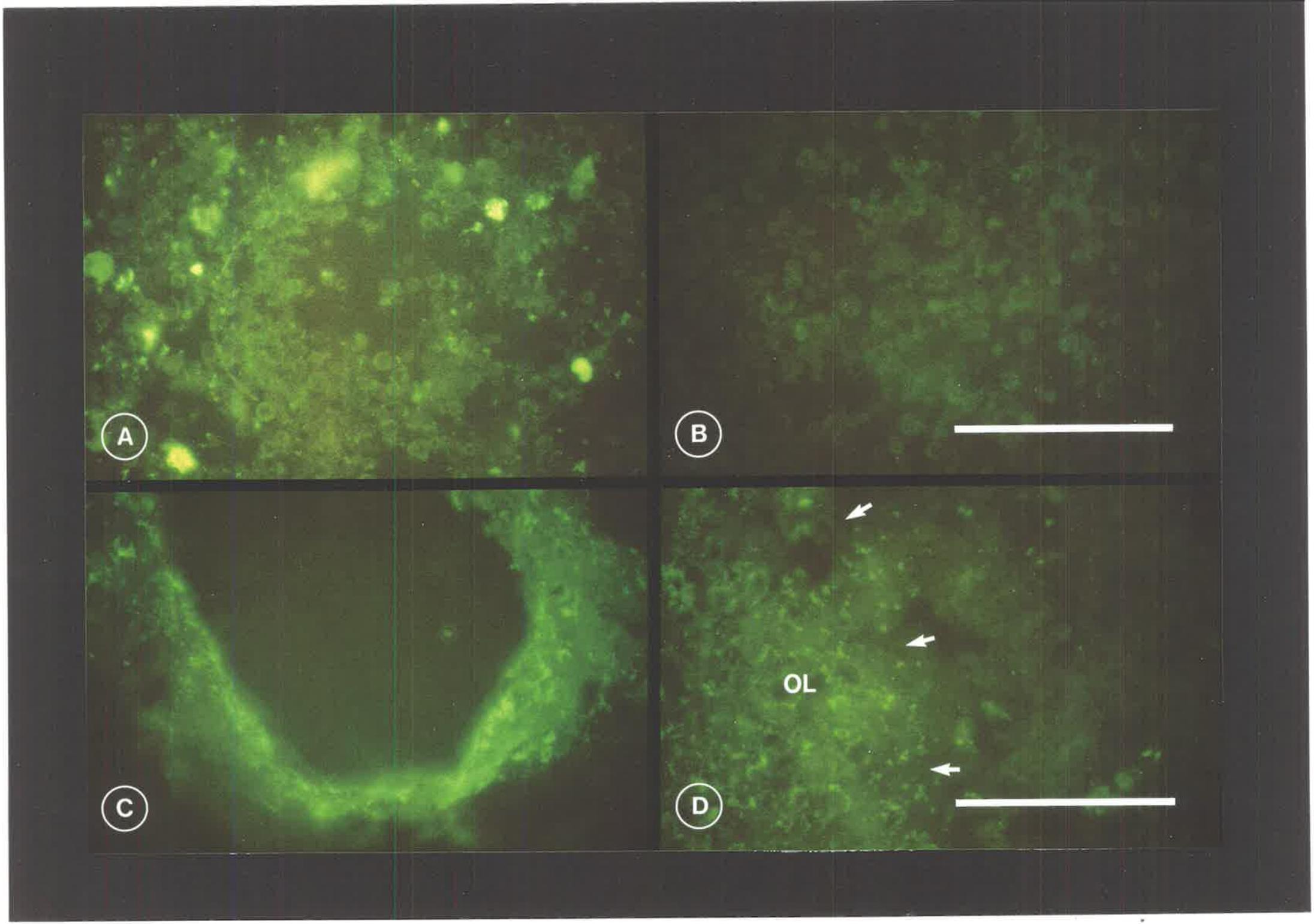

a
b
c
0.1 AU
d
e
f
g
h
j
k
l
m

Figure 4.4

(A) and (B) Immunofluorescent staining of smear preparations from SBV-infected and uninfected pupae, respectively. Note presence of specific staining reaction in (A), and absence in (B). Both at identical magnification; bar = 100 µm.

(C) and (D) Immunofluorescent staining of SBV-infected specimens prepared by cryomicrotomy 80 hr post-inoculation. (C) shows SBV-specific fluorescence in tracheal epithelial tissue. (D) shows SBV-specific fluorescence in the optic lobe (OL) of the brain; arrows delineate approximate margin of the optic lobe, scattered fluorescence in remainder of micrograph is from virus in hypopharyngeal gland cells and cells of the fat body. Both at identical magnification; bar = 100 µm.

All preparations fixed in acetone and stained with FITC-conjugated rabbit anti-SBV immunoglobulin.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OL

hypopharyngeal gland. Forty hours after SBV inoculation, fluorescence could be observed in sections from the hindgut, hypopharyngeal and salivary glands, brain, thoracic musculature, epidermis, tracheal epithelium, tracheal end cells, and in fat, and was also seen in haemocytes, and in haemocoelic spaces. Sections prepared from infected pupae after an incubation period of greater than 40 hr showed higher levels of fluorescence, but no further increase in tissue distribution. Figure 4.4 (c) and (d) show SBV-specific fluorescence in sections of tracheal epithelium and brain, prepared from pupae in which the virus had multiplied for 80 hr.

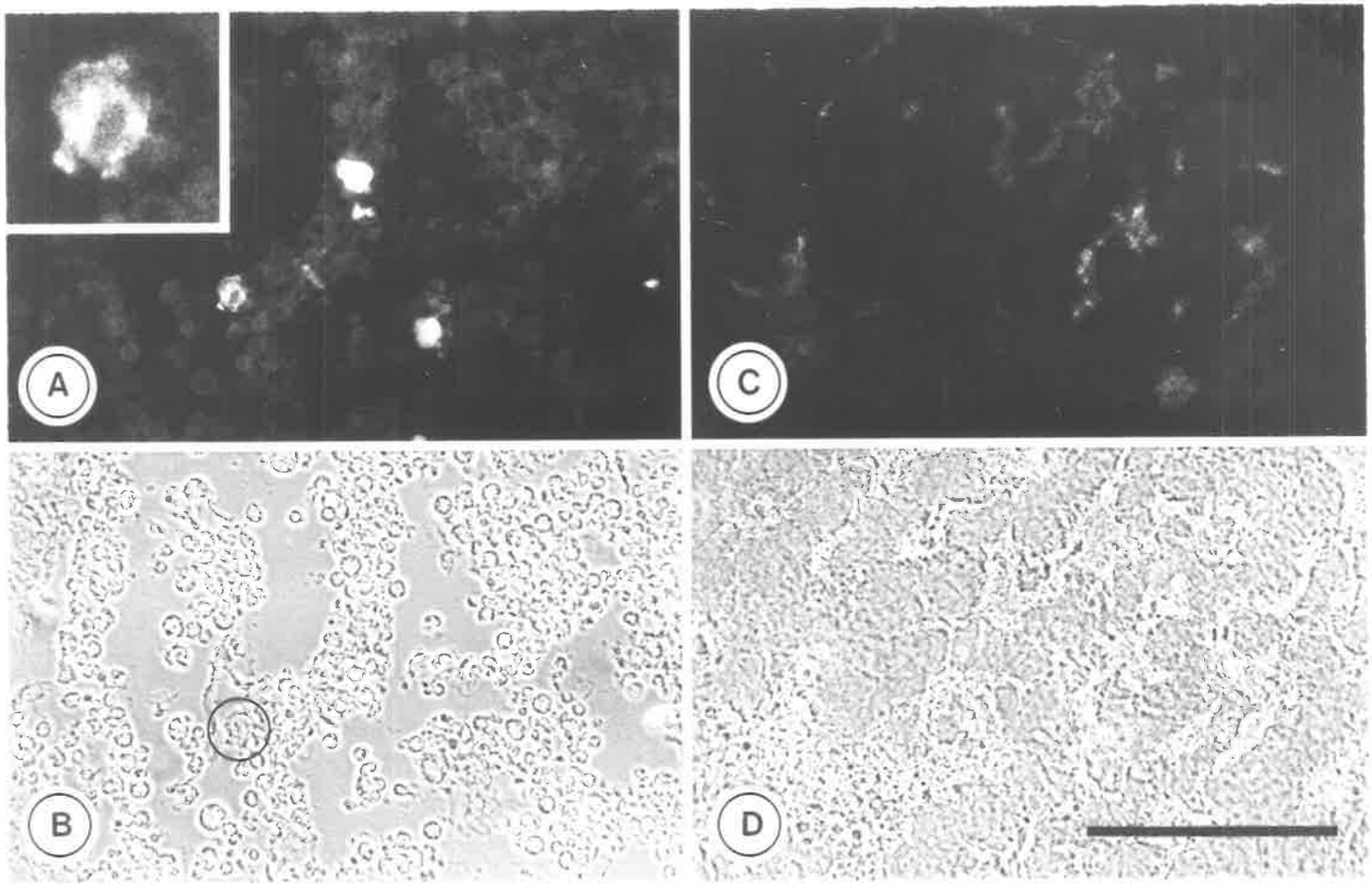
Interpretation of fluorescent staining in sections from pupae infected with KBV was difficult. Fluorescence could not be seen in specimens prepared from pupae in which KBV had multiplied for less than 20 hr, but with incubation times of 20 hr or more, fluorescence appeared restricted to spaces within the tissues, rather than being distributed within specific cell types. This fluorescence was, at best, diffuse [see Figure 4.5 (c) and (d)], and was interpreted as being due to staining of viral particles released into the haemolymph during rupture of necrotic cells (see Sections 5.2.2 and 5.3).

4.3.3 Acridine Orange staining

Tissue sections stained with acridine orange were used for independent validation of the results obtained by immunofluorescent staining of SBV, and as a second attempt to determine the sites of KBV multiplication in pupal tissue.

Figure 4.5

Fluorescence and bright field illumination of specimens prepared by cryomicrotomy. (A) and (B) show haemocytes and remnants of fat body cells prepared from an SBV-infected pupa 20 hr post-inoculation. Fluorescence is restricted to haemocytes, and the inset to (A) shows its subcellular distribution in one of these [circled in (B)]. Although fluorescence is clearly visible close to the nucleus, in a position analagous to that of the vesicular structure shown in Fig. 4.7, fluorescence at the cellular margin might result from pinocytotic uptake of virus. (C) and (D) show an area of thoracic musculature from a KBV-infected pupa 20 hr post-inoculation. Note the diffuse nature of the fluorescence, and its localisation within haemocoelic spaces. Each at identical magnification; bar = 100 μm.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Areas of bright orange-red staining, indicative of sites of RNA accumulation (Armstrong, 1956), were readily discernable in sections prepared from SBV-infected pupae [Figure 4.6 (a) and (b)]. Such accumulations, assumed to reflect the presence of viral RNA, were not seen in tissues of uninfected pupae [Figure 4.6 (c)]. By use of this method SBV was identified in the foregut and hindgut, hypopharyngeal and salivary glands, the musculature surrounding the midgut, in the epidermis, tracheal epithelium, fat, haemocytes, and in peripheral areas of nerve ganglia, in what were considered to be glial cells.

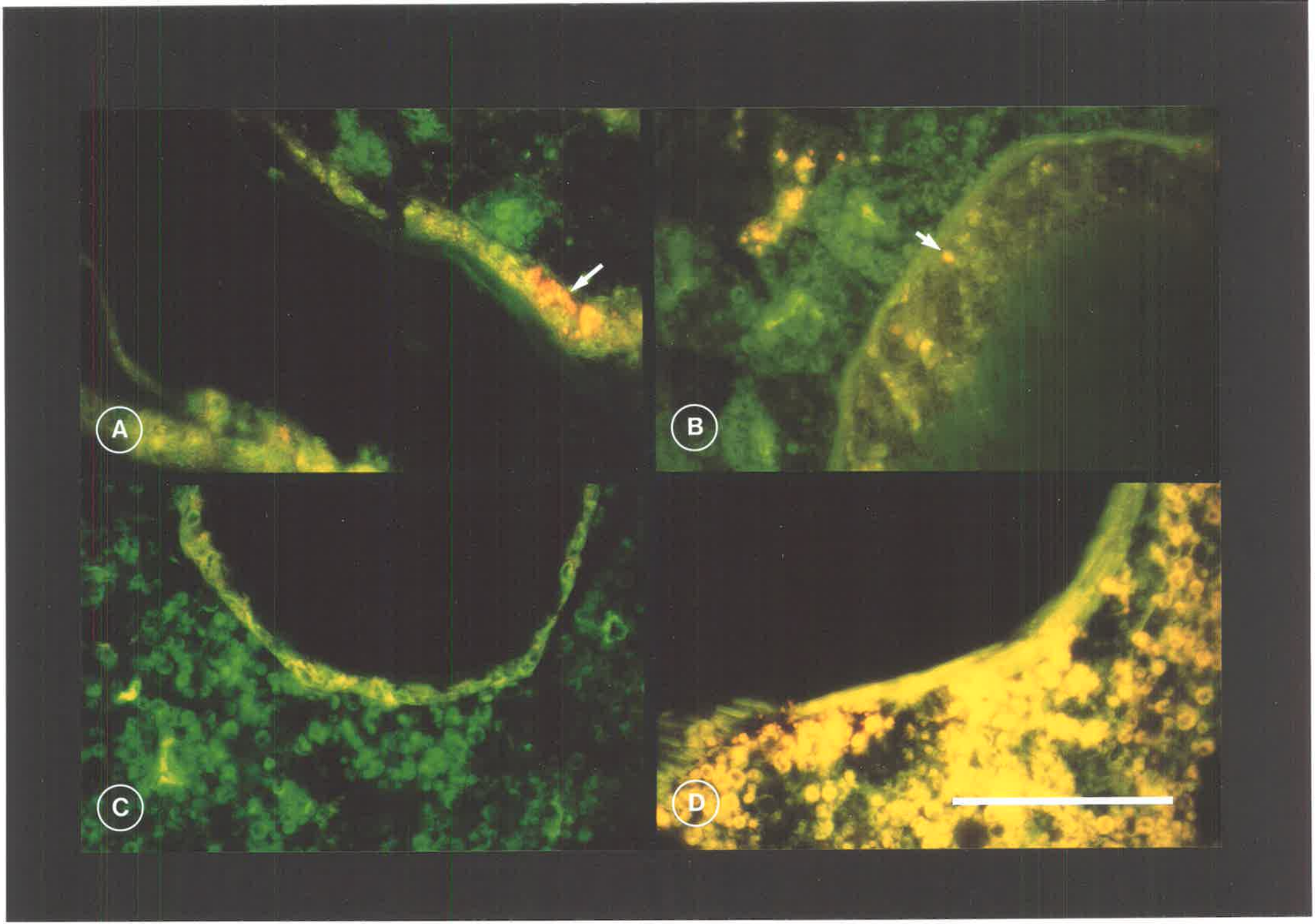
As with fluorescent antibody staining, interpretation of staining patterns in KBV-infected tissue proved difficult. Infected specimens were found to have staining characteristics which differed from those of SBV- or uninfected specimens. Figure 4.6 (d) illustrates the generalised orange/yellow appearance of KBV-infected specimens, and shows the unsuitability of this staining method for use in the determination of the specific sites of KBV multiplication.

4.3.4 Electron microscopy

Preliminary experiments on subsamples of tissue, prepared with or without pancreatic ribonuclease treatment, confirmed that the technique of Hatta and Francki (1981), originally described to allow differentiation between plant virus particles and host ribosomes, was applicable to studies of these two small RNA viruses of insects. Accordingly, RNase treatment was used routinely in specimens prepared for investigation of the distribution, multiplication and histopathological effects of the viruses. Because such studies are

Figure 4.6

Acridine orange staining of pupal tissue specimens. (A) shows an area of orange-red staining (presumed to indicate a site of accumulation of viral RNA) in abdominal epidermal cells (arrow) of an SBV-infected pupa, prepared 40 hr post-inoculation. (B) shows a similar staining reaction (arrow) within a thoracic ganglion of an SBV-infected pupa, prepared 40 hr post-inoculation. (C) shows a preparation from an uninfected pupa; note the absence of the orange-red staining reaction seen in (A) and (B). (D) shows tracheal epithelium and fat body cells of a KBV-infected pupa, prepared 24 hr post-inoculation; the general yellow/orange staining reaction is unlike that seen in preparations from either SBV-infected or uninfected pupae. Each at identical magnification; bar = 100 µm.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D

inextricably linked, micrographs supporting the results presented below are included in later stages of the thesis, in Sections 4.4 and 5.3.

Observations on the distribution of virus in tissues from SBV-infected pupae further confirmed the results obtained by other methods, described in Sections 4.3.2 and 4.3.3, and are summarised in Section 4.3.5.

Structures associated with the multiplication of KBV (see Section 4.4.2) were found in sections prepared from the foregut, hindgut, alimentary canal musculature, epidermis, and tracheal epithelium, and in haemocytes, oenocytes and tracheal end cells. Sections prepared from tissues of the central nervous system were examined closely, but neither these, nor other tissues not specifically mentioned, appeared to support the growth of KBV.

4.3.5 Discussion

The distribution of SBV, as determined in this study, and summarised in Table 4.6, agrees with results from previous investigations on adult honey bees (Lee and Furgala, 1967a ; Bailey, 1969 ; Mussen and Furgala, 1977), and confirms the widespread occurrence of this virus within the host. The work presented in this section also extends the knowledge of SBV biology by providing the first report of infection in the foregut and hindgut. Previously, SBV spread during communal feeding and nursing behaviour was thought to originate in the hypopharyngeal and mandibular glands (see Mussen and Furgala, op. cit.) ; while these sources are undoubtedly important, it must now also be considered that virus might be transmitted from ruptured or sloughed

Table 4.6 : Summary of the distribution of SBV and KBV in tissues of honey bee pupae, based on results described in Sections 4.3.2 to 4.3.4.

Tissue	SBV	KBV
Foregut epithelium	+	+
Midgut epithelium	-	-
Hindgut epithelium	+	+
Alimentary glands	+	-
Muscle	+	+
Fat body	+	-
Haemocytes	+	+
Oenocytes	-	+
Epidermis	+	+
Tracheal epithelia	+	+
Nervous system	+	-

off foregut epithelial cells. For many viral diseases of insects the hindgut, too, is an important site of infection, since from there, virus can be continually disseminated in faecal material (Moore and Tinsley, 1982). Although this tissue contained high concentrations of SBV, such infection is probably of only slight importance for the spread of bee viruses, since adult bees defaecate only outside the hive, usually while in flight (Michener, 1974).

The pattern of SBV spread, revealed by fluorescent antibody staining, was much as expected, given the relative importance already assigned to various tissues by the authors cited above. Thus, the early appearance of virus in the hypopharyngeal glands, as detected here, agrees with the important disseminatory role of these organs (Bailey, 1969). While fluorescence microscopy showed haemocytes to be the first cells in which virus could be identified, it is difficult to interpret this finding. At later stages of infection, haemocytes examined by electron microscopy were found to contain structures typical of SBV multiplication (see Section 4.4.1), but it must be considered possible that SBV detected 20 hr post-infection might have been scavenged and concentrated from inoculum in the haemocoel.

All three methods of examination proved to be applicable, and essentially in agreement, when used for investigation of SBV distribution. However, as described earlier, only electron microscopy could be used to study the distribution of KBV. Failure of the fluorescent antibody and acridine orange staining techniques is thought to reflect the cellular response to KBV infection, described in detail in Chapter 5. Lysis of KBV-infected cells is presumed to release large amounts of cellular contents and virus particles into the haemocoel,

causing both techniques to show widespread staining in areas in which KBV does not multiply.

Observations of KBV distribution in pupal tissues are summarised in Table 4.6, but since they are based on only one method of examination, these results should be treated with caution. The distribution pattern presented here shows many similarities with those recorded for other small RNA viruses of insects, including SBV. The most notable difference is the apparent absence of KBV from the nervous system. While use of electron microscopy alone has led to at least one record of false negative results from nervous tissue (Furgala and Lee, 1966), in this study extensive examination of samples of brain and ganglia failed to demonstrate the presence of KBV.

Distribution of virus in insect tissue has been determined for three other small RNA viruses which cause symptoms of paralysis - acute and chronic paralysis of honey bees (Lee and Furgala, 1965b ; Bailey and Milne, 1969) and cricket paralysis (Reinganum et al., 1970). In each case the virus has been found to replicate in nervous tissue, and this has been assumed to be responsible for paralysis of the host. KBV is clearly distinguished from these viruses by the fact that it, too, can cause host paralysis (Bhambure and Kshirsagar, 1978), but apparently without infecting the nervous system. Results from the further investigation of this topic are presented in Section 5.4.

4.4 Intracellular structures associated with viral multiplication

4.4.1 Sacbrood virus

Three distinct and unusual types of cytoplasmic inclusion were consistently found in cells infected by SBV. On the basis of their presumed homology with structures described by previous workers (Lee and Furgala, 1967a ; Friedmann and Lorch, 1984), these have been identified as (i) vesicular structures, (ii) various types of particle aggregates, and (iii) complex myelin-like membranous structures. More detailed descriptions of these inclusions now follow.

The vesicular structure (VS), was generally found in close proximity to the nucleus, often within a depression in the surface of that organelle [see Figure 4.7 (a)] ; from this site the distal regions of the structure could extend almost to the cell membrane. The VS was not membrane bound, but rather, consisted of an amorphous, electron-dense matrix surrounding a loose aggregation of membranous vesicles. These vesicles had a diameter of 100 to 800 nm, and many contained fine, interwoven strands of fibrillar material [Figure 4.8 (c)]. Inspection of the surrounding matrix showed that a large proportion of its volume apparently consisted of empty virus-like particles [VLP; see Figure 4.8 (a and b)]. A relatively small number of complete virus particles were usually also present, sometimes distributed around the circumference of the membranous vesicles.

Large numbers of empty VLP could commonly be seen in the nuclei of infected cells [Figure 4.7 (a) arrowheads]. As discussed later (Section

Figure 4.7

(A) An SBV-induced vesicular structure (VS) in an epithelial cell from the hindgut of an infected pupa, prepared 72 hr post-inoculation. Note proximity of the VS to the cell nucleus (arrows show nuclear membrane), and the presence of virus-like particles within the nucleus (arrowheads). Bar = 1 µm.

(B) SBV particles and large numbers of empty virus-like particles in the cytoplasm of a tracheal end cell, 72 hr post-inoculation. Bar = 1 µm.

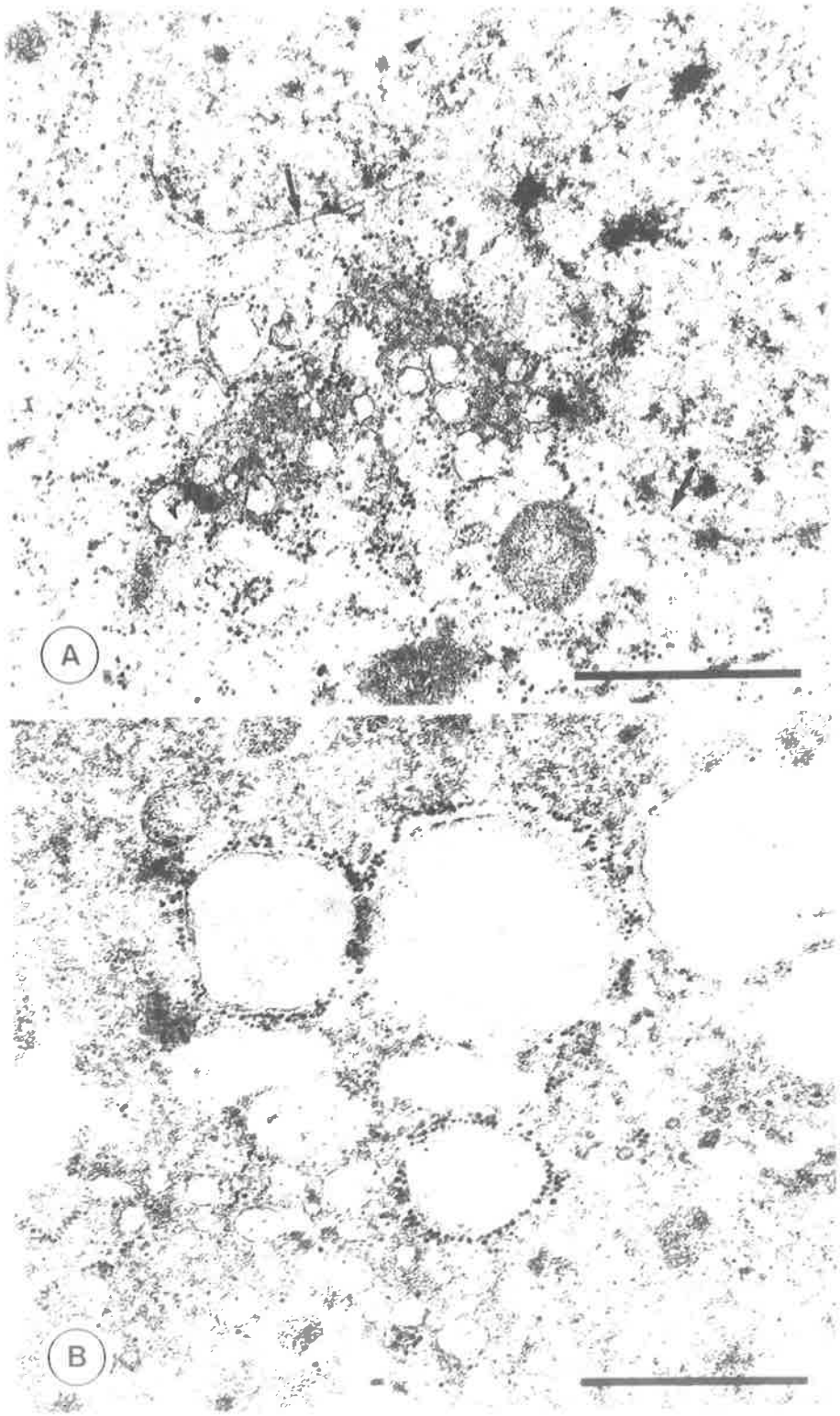
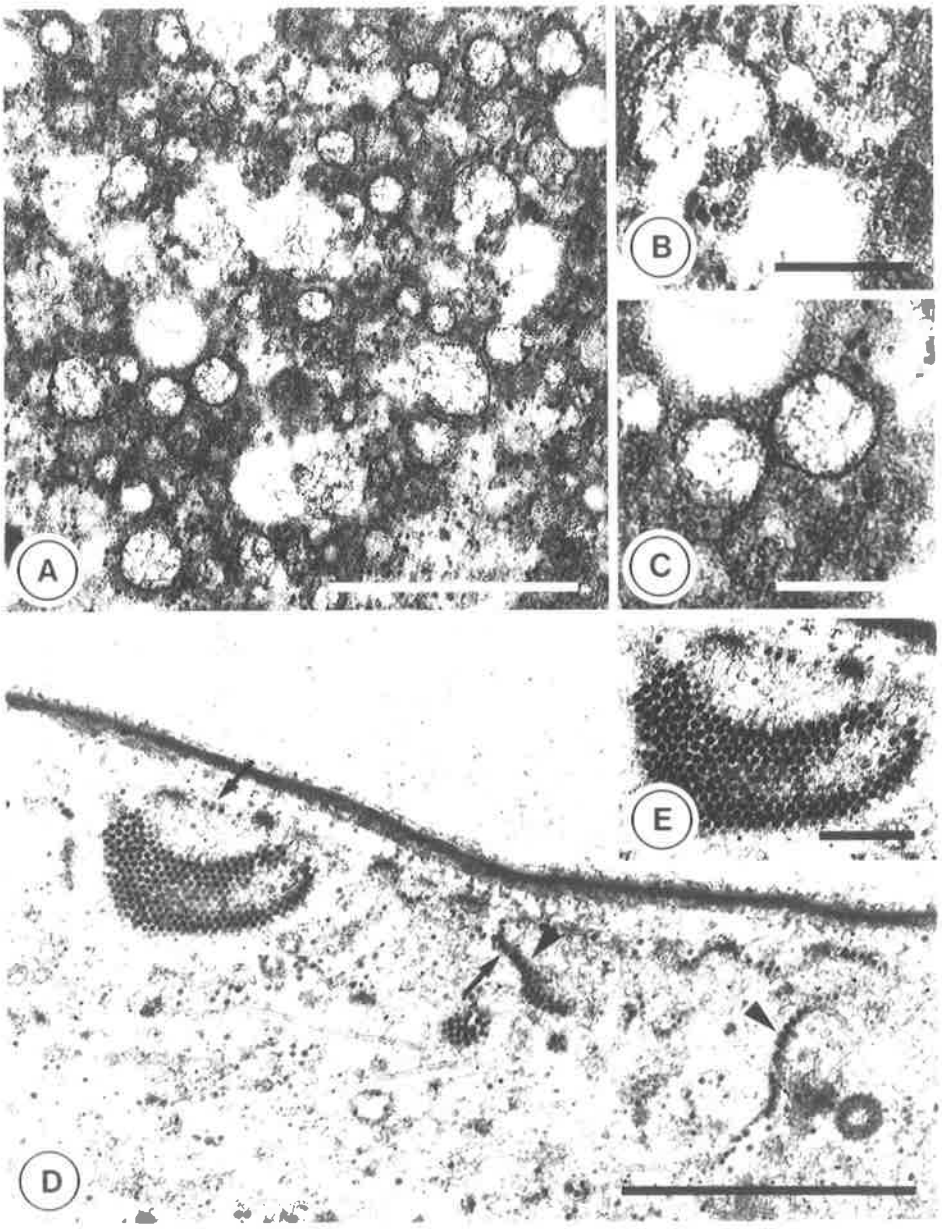

A
B

Figure 4.8

(A) Part of an SBV-induced vesicular structure in an hypopharyngeal gland cell, prepared 72 hr post-inoculation. Note membranous vesicles with fibrillar contents, surrounded by an electron-dense matrix containing complete SBV particles and empty virus-like particles. Selected areas of (A) are shown at higher magnification in (B) and (C). Bar = 1 μm (A) or 250 nm (B) and (C).

(D) Arrangement of SBV particles within limiting membranes in an epithelial cell from the hindgut of an infected pupa, prepared 72 hr post-inoculation. Note clear single layer arrangement (arrows), oblique view showing partially superimposed particles (arrowheads), and array of particles, presumed to show packing arrangement between membranes. (E) shows array of SBV particles at higher magnification. Bar = 1 μm (D) or 250 nm (E).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D
E

4.4.3) this organelle is thought to be a site of VLP accumulation, rather than a site of synthesis.

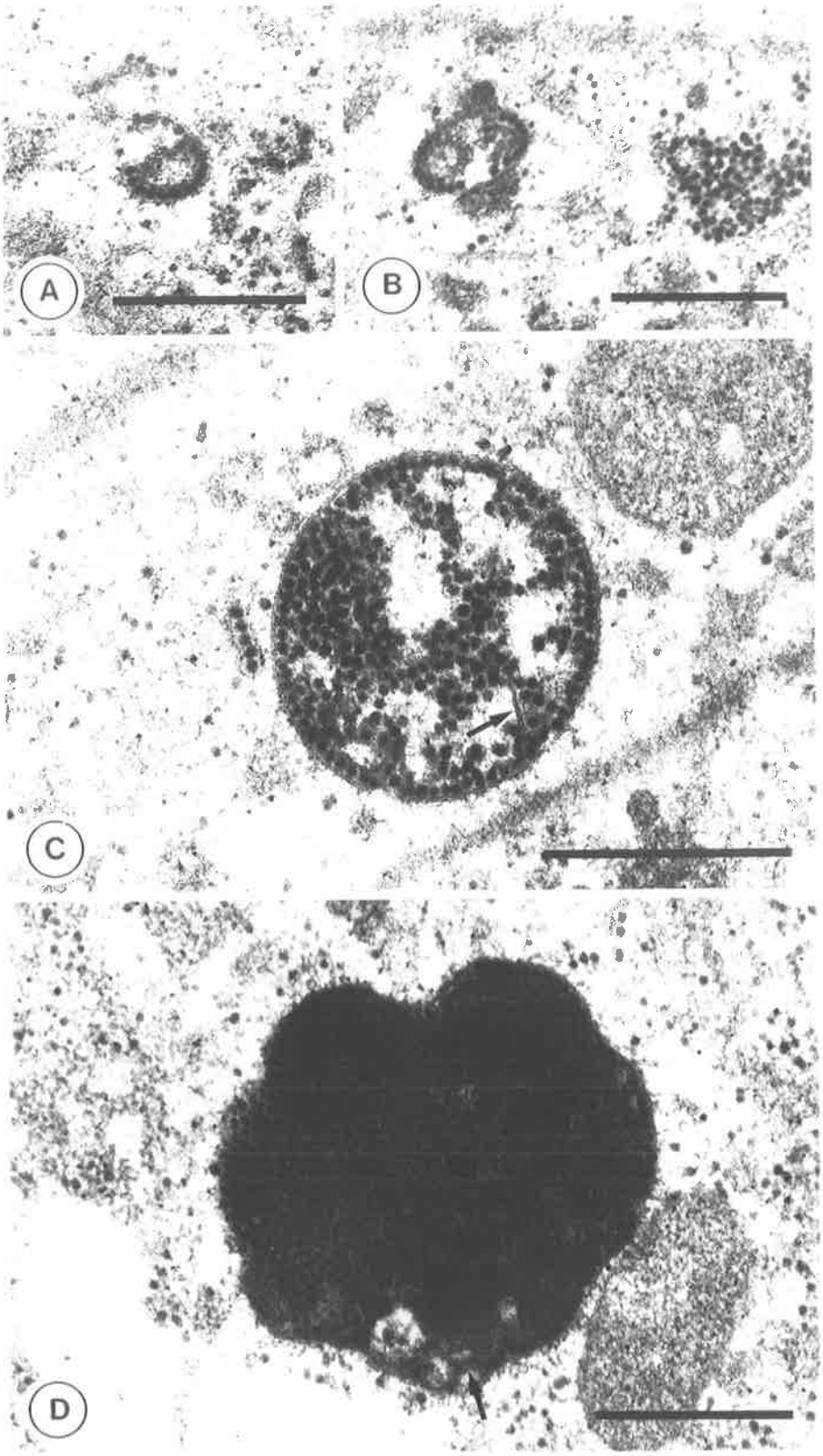
Mature particles of SBV were arranged in one of at least four different configurations within an infected cell. In addition to being distributed singly within the cytoplasm, particles were seen in random aggregates, as in Figure 4.9 (b), and within two types of membrane-bound structure.

Figure 4.8 (d) illustrates one of the membrane-bound virus associations, in which particles were arranged in a single layer between two limiting membranes. This micrograph shows three different aspects of such an assemblage, namely, a cross-sectional view showing a single row of particles (arrows), an oblique view, showing a blurred image of partially superimposed particles (arrow heads), and a 'face-on' view, which shows the close packed arrangement of particles between the membranes [left midground; see also 4.8 (e)].

Figure 4.9 shows examples of the second, more common type of membrane-bound structure, subsequently referred to as a viral inclusion body (VIB). It should be noted here that the method of formation of VIB's is uncertain, so that differently sized structures shown in this figure cannot necessarily be interpreted as representing different stages in a developmental sequence (see Section 4.4.3 for further discussion of this point). VIB's are not considered to be homologous with the membrane-bound vesicles of the viroplasmic region, since they are neither surrounded by a matrix, nor necessarily found in aggregations with other similar structures.

Figure 4.9

Viral inclusion bodies (VIB) of SBV in hindgut cells from infected pupae, prepared 72 hr post-inoculation. Note that double membranes surround all VIB's and may also be present within the structure [(C) and (D), arrows]. Individual virus particles are difficult to distinguish in (D) due to the presence of finely-grained electron-dense material within the VIB. In each micrograph bar = 500 nm.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In all cases VIB's were bounded by a double membrane, and in partially filled structures apparently identical membranes could be seen within the central region of the body [Figure 4.9 (c), arrow]. Virus particles were usually arranged regularly around the circumference of the VIB, but within the central region seemed to be randomly distributed in conjunction with other finely-grained electron-dense material. Viral inclusion bodies ranged in diameter from about 200 nm to over 1 μm, as shown in Figure 4.9 (a) and (d), respectively.

Figure 4.10 shows three examples of complex structures of myelin-like material in the cytoplasm of cells infected with either SBV alone [(a) and (b)], or SBV and KBV together [(c), see also Chapter 6]. Such inclusions were composed of intricate stacks and whorls of double membranes, and seemed always to contain circular (presumably spherical) electron-dense areas, considered to represent deposits of lipid material. They were often found in association with cytoplasmic aggregates of virus particles, as in Figure 4.10 (b), and themselves often contained small to vast numbers of mature particles. The possible role(s) of these membranous inclusions in the infective process are discussed in Section 4.4.3.

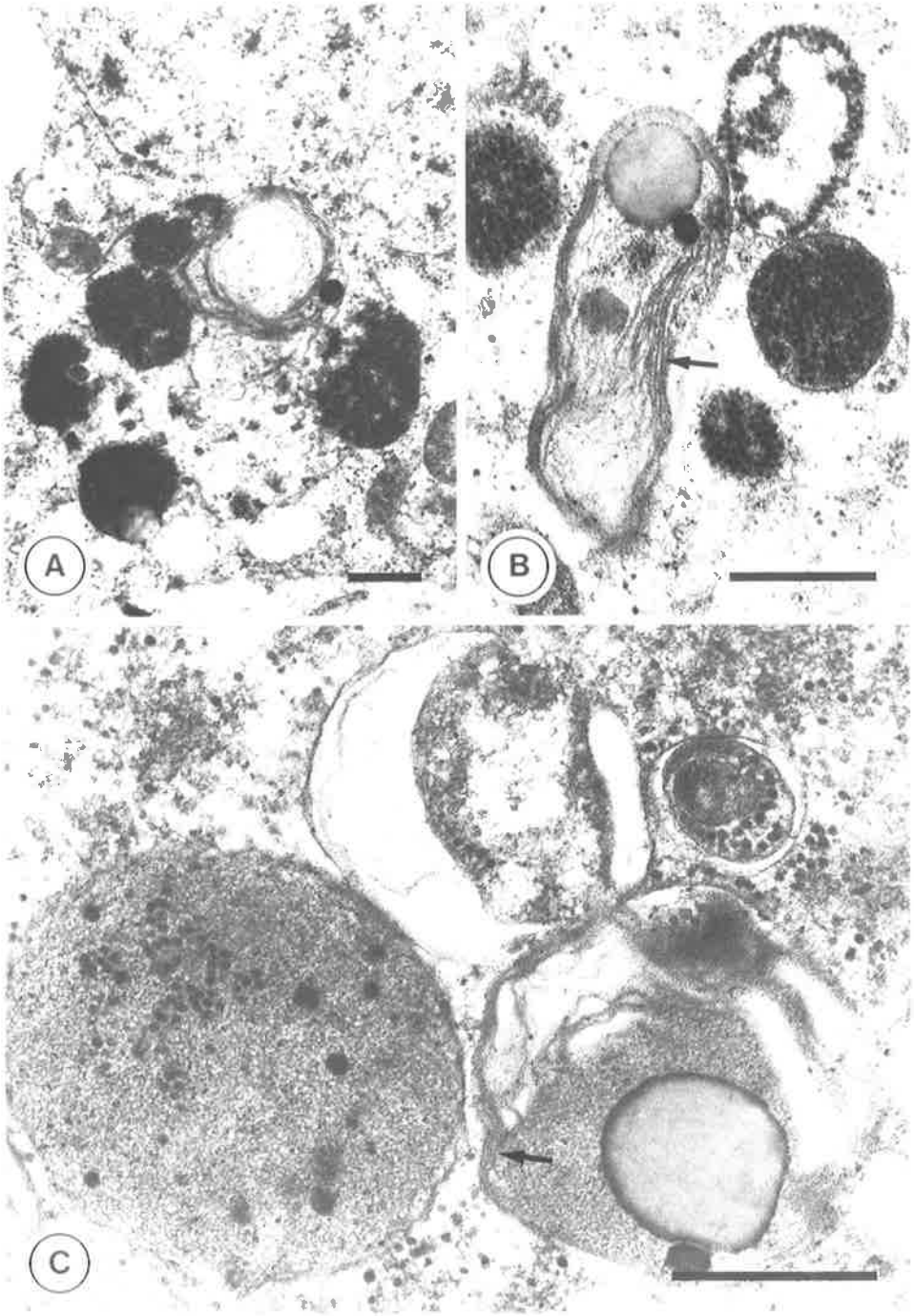
The structures described in this section were seen only in cells of pupae infected with SBV, and were not observed in cells of healthy pupae processed in parallel with infected specimens.

4.4.2 Kashmir virus

Examination of infected cells showed that KBV induced only one type of cytoplasmic inclusion, most simply described as a membrane-bound

Figure 4.10

Myelin-like structures in the cytoplasm of hindgut epithelial cells from SBV-infected pupae, prepared 72 hr post-inoculation [(A) and (B)], and a foregut epithelial cell from a double-infected pupa [(C), SBV with KBV, 72 and 48 hr post-inoculation, respectively]. Note the prominent lipid deposits and the stacks and whorls of double membranes [(B) and (C), arrows]. As shown, these membranes enclose numbers of virus particles; in (A) several large masses of SBV particles (appearing as dark, finely-grained bodies) are associated with the structure. In each micrograph, bar = 500 nm.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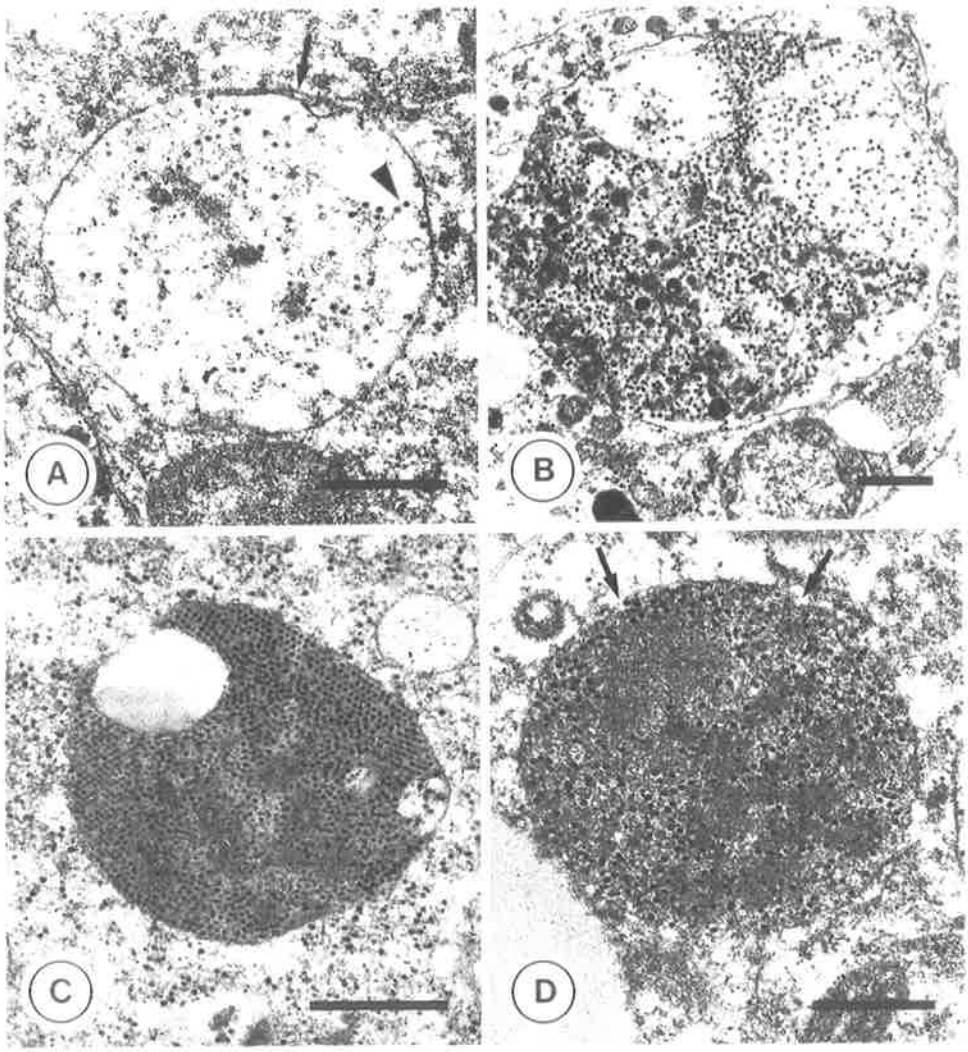
vesicle. Such vesicles ranged in diameter from several hundred nm to several μm, and showed no evidence of localisation or aggregation within the cell, being distributed apparently at random throughout the cytoplasm.

Many vesicles showed evidence of internal membrane proliferation, apparently as a result of continued growth of the internal layer of the double boundary membrane [see Figure 4.11 (a), arrow]. While large structures were sometimes formed in this manner, no functional differences were detected between 'simple' and 'complex' vesicles, and in both cases apparently identical series of developmental stages were present. Figure 4.11 shows examples of these stages arranged in a putative developmental sequence.

Figure 4.11 (a) shows a structure interpreted as a young vesicle, which contains empty virus-like particles, complete virus particles, some thin fibrils (arrow head), and a small amount of finely-grained electron-dense material. Similar contents are present in the ingrowths of the vesicle shown in 4.11 (b), while in the main body of the structure there is evidence of continued condensation of electron-dense material, and an increase in the ratio of mature/empty particles. Figure 4.11 (c) is considered to represent a mature vesicle in which virus replication is essentially complete. Note that the boundary membrane is still intact, that few empty particles are present, and that the particles are arranged within an almost homogeneous matrix. Finally, micrograph (d) shows a 'senescent' vesicle in which the boundary membrane has ruptured (arrows), facilitating the release of mature virus particles, and other enclosed material, into the cytoplasm of the cell.

Figure 4.11

Membrane-bound cytoplasmic inclusions associated with KBV replication, arranged in a putative developmental sequence. Inclusions shown in (A), (B) and (D) were from foregut epithelial cells, and in (C) from a tracheal epithelial cell, each prepared 40 hr post-inoculation. (A) shows a vesicle at a putative early stage of development; note the empty virus-like particles, complete particles and fibrillar material (arrowhead). Proliferation of the internal layer of the double membrane [(A), arrow] is thought to result in formation of complex structures such as that shown in (B). (C) shows a mature vesicle with an intact external membrane surrounding virus particles in an essentially homogenous matrix, while (D) shows a 'senescent' vesicle apparently disintegrating (arrows) to release mature KBV particles, and matrix material, into the cytoplasm. In each micrograph bar = 500 nm.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4.4.3 Discussion

The vesicular structure induced by SBV has not previously been described, although Mussen and Furgala (1977) alluded to its presence in infected hypopharyngeal gland cells of adult bees. In many respects this structure is similar to the aggregations of smooth membrane seen in cells infected with vertebrate picornaviruses such as Theiler's murine encephalomyelitis virus (TMEV; Friedmann and Lorch, 1984), although there are also some significant differences. Thus, while large amounts of picornavirus capsid protein are known to accumulate in such areas (Godman, 1973), the assembly of large numbers of empty particles, as seen for SBV, appears to be unusual. The possibility exists that these particles are experimental artefacts caused by RNase digestion, but this seems unlikely, since that treatment did not affect the SBV sedimentation profile (Figure 4.3).

Both viral coat proteins and arrays of empty capsids have previously been reported in nuclei of cells infected with vertebrate picornaviruses (Anzai and Ozaki, 1969 ; Beinz et al., 1982 ; Harrison et al., 1971). Similar observations have been made in nuclei of plant cells infected with members of the Tymovirus group (Matthews, 1981), and observations on one such virus (Okra mosaic virus) suggest that coat proteins enter the nucleus as monomers and oligomers, and are then assembled as empty particles (Marshall and Matthews, 1981). While the present study contains no experimental evidence on the site of synthesis or assembly of empty VLP in nuclei of SBV infected cells, it seems likely that for this virus, too, the nucleus represents a site of accumulation of viral protein, rather than of synthesis. The

intranuclear presence of these VLP appears to distinguish SBV from most, if not all, of the small RNA viruses of insects.

Structures similar to the SBV-induced membranous vesicles have been recognised in many types of cell infected with RNA viruses, and in several cases there is good evidence to suggest that their fibrillar contents are double-stranded (ds) RNA (de Zoeten et al., 1974 ; Hatta and Francki, 1978). Again, it seems likely that the same is true for the contents of SBV-induced vesicles. Lee and Furgala (1967b) published a micrograph from an SBV-infected cell, showing fibrillar material within a matrix of viral particles, and suggested that this, too, was dsRNA. However, the identity of this structure seems to be questionable, since it was apparently isolated within a mass of mature particles ; in this study, vesicles associated with SBV multiplication were always found in aggregations, surrounded by empty protein shells.

Since the vesicular region almost certainly contains viral RNA, and is known to contain both viral protein (as shown by formation of empty VLP), and low numbers of mature particles, it is assumed to represent the site of viral synthesis and assembly within the cell. On this basis, the inclusion bodies observed elsewhere in the cytoplasm are thought to represent secondary aggregates of viral particles.

Friedmann and Lorch (1984) noted that particles of the TO strain of TMEV were usually found between two membranous sheets, and that depending on section orientation, this gave rise to various 'single file' and monolayer appearances. The size and shape of such structures, as published, are very similar to those formed by SBV, as shown in Figure 4.8 (d) and (e). In both cases the diameter and sheet-like nature

of the enclosing membranes suggest that they are not formed from cellular microtubules, and while their exact origin is not clear, Friedmann and Lorch (op. cit.) suggested that those of TMEV might be part of the membrane system which forms the vesicular structure. Given the other similarities described above, it seems likely that such an interpretation is equally applicable to those of SBV.

It is tempting to speculate that a multilayered circular folding, or 'rolling', of such sheet-like configurations gives rise to the VIB structures illustrated in Figure 4.9. Such a process would account for a number of the features shown by these bodies, including their generally circular shape, the precise orientation of particles around their circumference, and the presence of surrounding and internal membranes [see Figure 4.9 (c)]. Further, if the internally-sequestered membranes were assumed to degrade, this model could also account for the uniform, finely-grained matrix which pervades these inclusions.

The complex myelin-like structures found to be associated with SBV infection have previously been described by Lee and Furgala (1967a). Those authors suggested that the association of SBV particles with these structures might be 'coincidental', or failing this, might be 'involved in the replication of virus'. Since that date, it has been shown that many viruses can induce the formation of such structures in the cells of both definitive and intermediate insect hosts (for example, Amyelois chronic stunt virus; Hoffman and Kellen, 1982 ; rice dwarf virus; Harris, 1979 ; maize rayado fino virus; Kitajima and Gamex, 1983) and also in infected plant cells (cowpea mosaic virus; Kim et al., 1974). The function(s) of such structures, like those induced by SBV, remain unknown, but suggestions have included on association with virus

replication (Kim et al., op. cit.), with host cell defence (Harris, op. cit. ; Hoffman and Kelly, op. cit.) or with degenerative cellular changes (Matthews, 1981).

In contrast to the various similarities displayed by stages of SBV replication, resemblances between growth of KBV and other viruses are difficult to find. While many viruses have been quoted or depicted as replicating within 'membrane-bound areas', there is generally insufficient detail to allow valid comparisons to be made.

One of few exceptions to this appears to be an un-named picorna-like virus of trematodes, in which replication is confined to cytoplasmic vesicles (Ip and Desser, 1984). In that virus, as for KBV, there is proliferation of membranes within the primary vesicle, and initial formation of empty particles, followed by the appearance of complete particles. These complete particles are subsequently released into the cytoplasm by rupture of the mature vesicles. Compared to KBV there are, however, a number of morphological dissimilarities, and given these, and the phylogenetic distance between the hosts, it is difficult to suggest the existance of any relationship between the two viruses. Similarly the calicivirus, Amyelois chronic stunt, while replicating in cytoplasmic vesicles which show complex internal membrane proliferation (Hoffmann and Kellen, 1982), also shows various morphological dissimilarities, suggesting that the resemblance between these two viruses is likely to be largely coincidental.

CHAPTER 5 : HISTOPATHOLOGICAL EFFECTS OF SACBROOD AND KASHMIR VIRUS INFECTIONS

5.1 Introduction

The preceding chapter contained descriptions of the multiplication of SBV and KBV, but made no mention of the consequences for the host cell. As a complementary approach, this chapter reports a study of the pathological changes associated with SBV and KBV infection, based on observations of alterations to the gross and ultrastructural morphology of host cells, and to some physiological characteristics of infected pupae.

The consequences of picornaviral infection have been studied in a number of cell culture systems, and have been found to vary markedly in different virus/host cell combinations. Thus, for example, hepatitis A virus has been shown to cause inapparent, persistent infection in human fibroblast cells (Vallbracht et al., 1984), while poliovirus infection of HeLa cells causes inhibition of host-cell RNA and protein synthesis (see Kaariainen and Ranki, 1984), changes in cell membrane permeability (Nair, 1981), alterations in ultrastructural morphology (Dales et al., 1965) and finally, cytolysis.

Studies of infection in whole animals show similar variation in severity, with status of infection varying from inapparent to life-threatening, as described in Chapter 3. Regardless of the host involved, such studies are far more complex to interpret than those done in cell cultures, since their outcome is determined by both the virulence of the virus and the susceptibility of the individual, and further complicated

by interaction between diseased and uninfected cells. These difficulties have prevented unequivocal explanation of 'the exact cause of damage to virally infected cells', and thus of 'the way in which such damage leads to ... the clinical symptoms of viral disease' (Pasternak and Micklem, 1981).

It is clear, however, that in most cases tissue damage can be categorised as being of 'direct' or 'indirect' origin. Mims (1982) describes 'direct' pathological damage as being cellular injury which results from the multiplication of an infectious agent, but which affects only the host cell. That author cites the sloughing of rhinovirus-infected nasal epithelial cells from the mucosal surface as an example of this type of damage. 'Indirect' pathological damage is also a consequence of microbial multiplication, but affects cells which are not themselves infected. Damage of this type may result from a number of causes, including the action of toxins produced by the infectious agent, or of inflamatory or cytolytic substances released from necrotic cells, and in vertebrates, as a result of the expression of the immune response (Mims, op. cit.).

There are relatively few studies which describe the direct pathological changes which accompany viral infection of invertebrate cells (Couch, 1981). As stated by Longworth (1978), for most small RNA viruses of invertebrates such knowledge is confined to casual observations made simultaneously with examination of host tissue for virus particle distribution.

In this study, infection with SBV could not be associated with any changes in cellular morphology or other physiological characteristics.

In contrast, infection with KBV was invariably found to cause major cytopathological damage, both to cells in which it replicated, and to tissues of the pupal nervous system, in which virus particles could not be observed. Investigations reported in Section 5.4 suggest that this latter observation may be the first recorded instance of indirect damage to invertebrate tissue caused by infection with a small RNA virus.

5.2 Light microscopy studies

5.2.1 Materials and methods

Light microscopy. Pupae were divided into head, thorax and abdomen, fixed for about 6 hr in alcoholic Bouin's fixative, then dehydrated in 95% and absolute ethanol for a total of about 24 hr. Samples were cleared for 6 hr in one change of xylene, then vacuum infiltrated in one change of paraffin wax (M.P. 60°C) over a period of 24 hr. Following embedding, sections were cut on a rotary microtome at thicknesses of between 4 and 7 μm.

After drying on microscope slides, sections were deparaffinised in xylene, hydrated in a descending alcohol series, and stained with Mallory's Triple Stain (Pantin, 1946). Sections were examined and photographed using a Leitz Orthoplan microscope fitted with a Wild MPS 45 Photoautomat system.

5.2.2 Results

Examination of paraffin-embedded tissue specimens showed that KBV infection was invariably associated with severe cellular damage in a

number of tissues. Sacbrood-infected specimens, however, did not differ in appearance from those taken from uninfected control pupae. For this reason the following descriptions of virally-induced tissue damage relate almost entirely to KBV, although in Figures 5.1 to 5.5, illustrations of SBV-infected tissues are also included.

Three types of cellular response could be recognised by light microscopy following infection with KBV. The most common was characterised by cellular rounding, retraction from neighbouring cells and/or membranes, and in many cases, by condensation of the nuclear material. This pattern was seen in tracheal epithelial cells, in haemocytes, in epidermal cells (as illustrated by those beneath abdominal plates in Figure 5.1) and in both epithelial cells and the surrounding muscle cells of foregut and hindgut (see Figure 5.2).

A second type of response, demonstrated only by oenocytes, has been illustrated in Figure 5.3. Once again marked contraction of the nucleus was evident, but in this case it was accompanied by the progressive formation of small lobules of cytoplasm, 'pinched off' from the circumference of the cell, but retained within the outer membrane. At later stages of infection (not shown), rupture of the surrounding membrane allowed these fragments of cytoplasm to disperse throughout the body cavity.

A third type of response was identified only in nervous tissue, where it was restricted to glial cells (including those in the perineurium), and to the chiasmata cells of the optic lobe, (see Figures 5.4 and 5.5). Because this response involved an almost total loss of cytoplasmic staining, light microscopy could provide little further

Figure 5.1

Morphology of abdominal epidermal cells from an uninfected pupa* (A), a KBV-infected pupa (B), and an SBV-infected pupa (C), each prepared 48 hr post-inoculation. Note that KBV infection (B) is associated with rounding and loss of contact between neighbouring epidermal cells, but that SBV infection produces no identifiable change in cellular morphology. Bouins fixative, Mallory's Triple Stain; each at identical magnification, bar = 150 µm.

* In legends to Figures 5.1-5.5 and Figure 5.11 the term 'uninfected pupa' denotes a control pupa inoculated with 2/3 Insect Ringers solution.

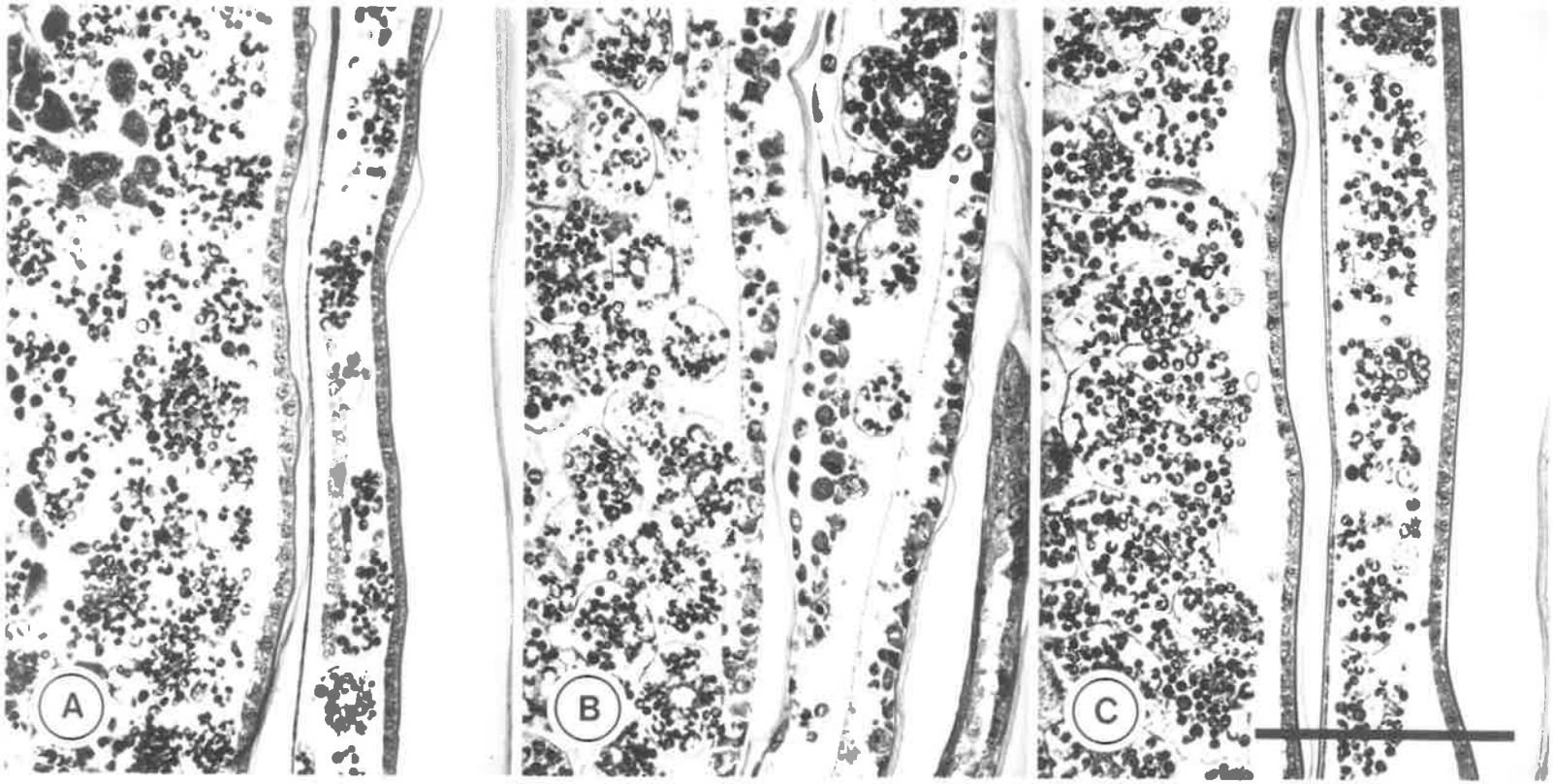

A
B
C

Figure 5.2

Morphology of hindgut cells from an uninfected pupa (A), a KBV-infected pupa (B), and an SBV-infected pupa (C), each prepared 48 hr post-inoculation. Note that KBV infection (B) is associated with rounding and loss of contact between neighbouring intestinal epithelial cells. Epithelial cells of the SBV-infected specimen shown in (C) are morphologically unchanged; this preparation was from a pupa slightly younger than those in (A) and (B). Bouins fixative, Mallory's Triple Stain; each at identical magnification, bar = 250 µm.

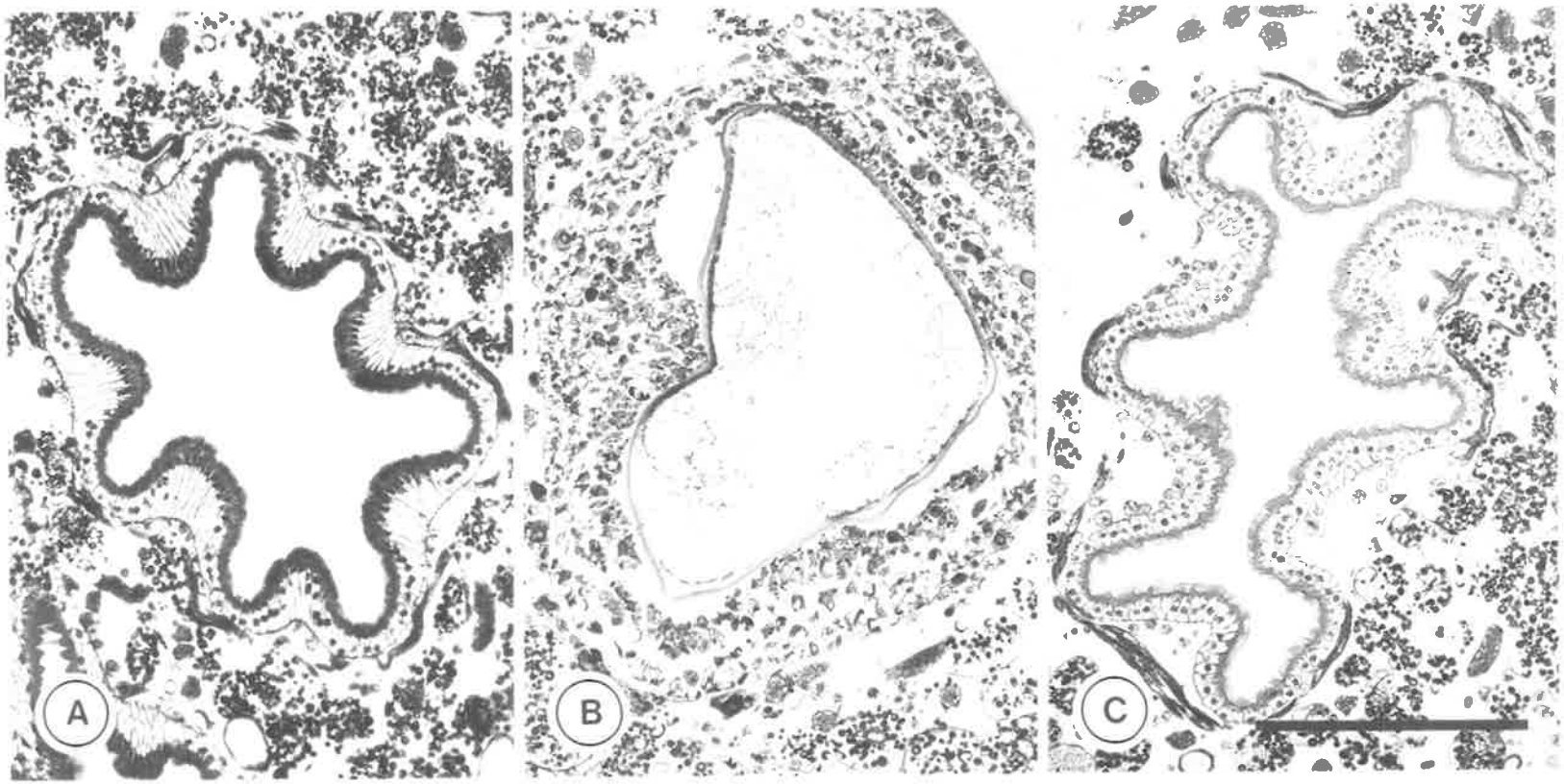

A
B
C

Figure 5.3

Morphology of oenocytes from an uninfected pupa (A), a KBV-infected pupa (B), and an SBV-infected pupa (C), each prepared 24 hr post-inoculation. Note that KBV infection is associated with contraction of the oenocyte nucleus [visible in (B) in centrally placed oenocyte, and in 'glancing' section in upper oenocyte (arrow)], and with formation of lobules of cytoplasm [(B), central cell], at this stage still retained within the cell membrane. No morphological changes are apparent in fat body cells in either the KBV- or SBV-infected specimens. Bouins fixative, Mallory's Triple Stain, each at identical magnification, bar = 150 μm.

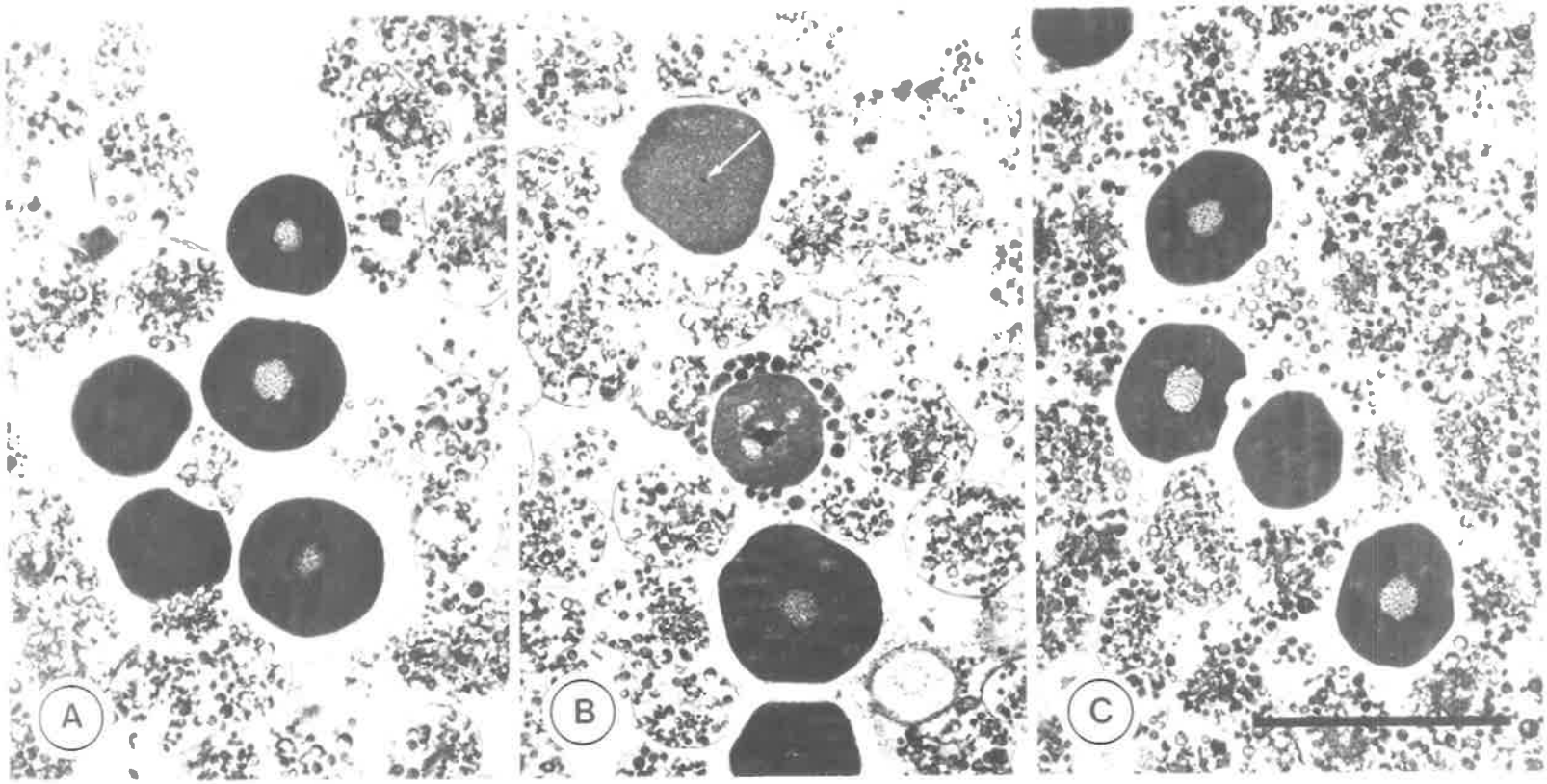

A
B
C

Figure 5.4

Morphology of cells of the proto- and deutocerebrum of an uninfected pupa (A), a KBV-infected pupa (B), and an SBV-infected pupa (C), each prepared 24 hr post-inoculation. Note that cellular damage induced by KBV infection - swelling and loss of cytoplasmic staining - is apparently restricted to glial cells. Bouins fixative, Mallory's Triple Stain; each at identical magnification, bar = 250 µm.

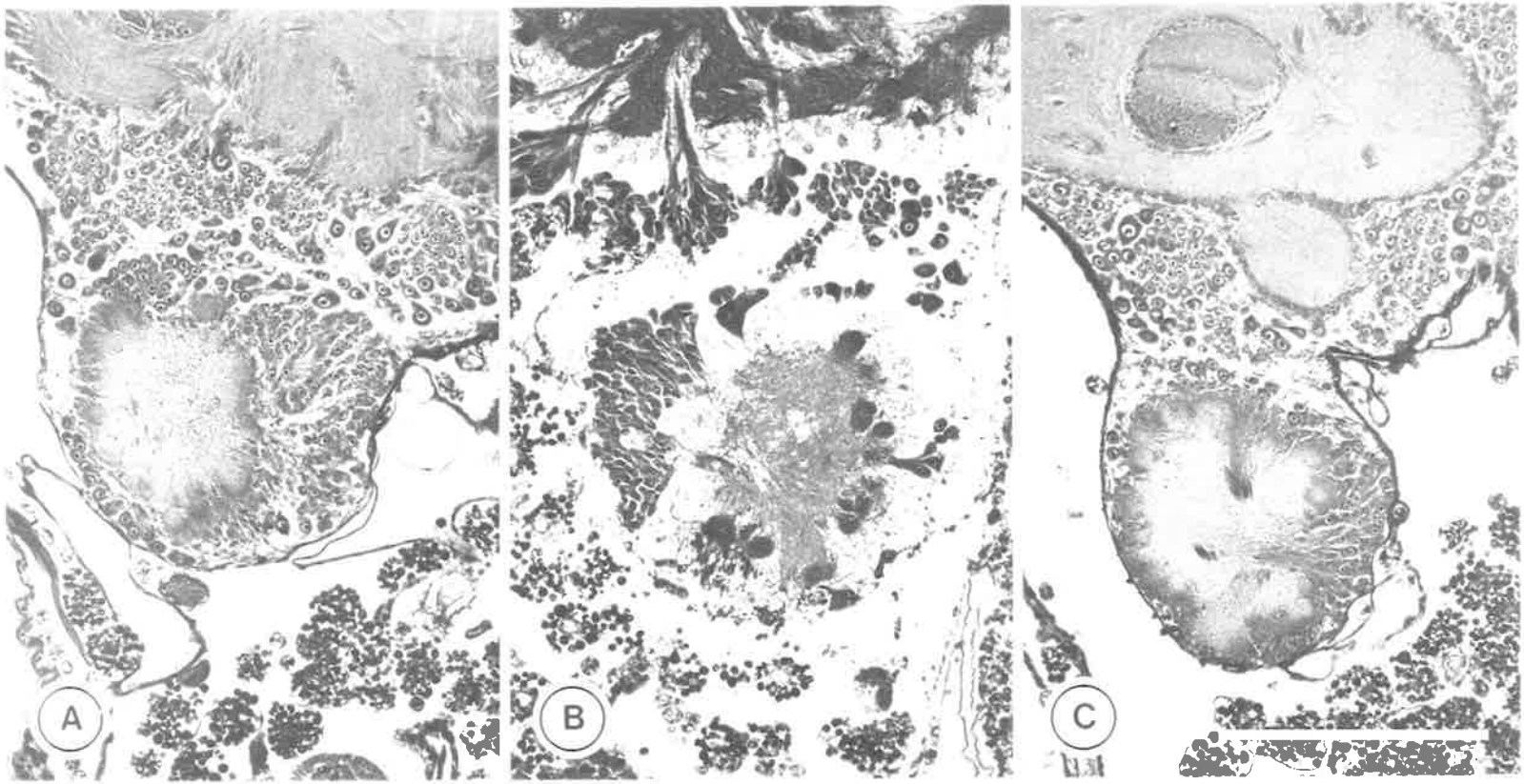
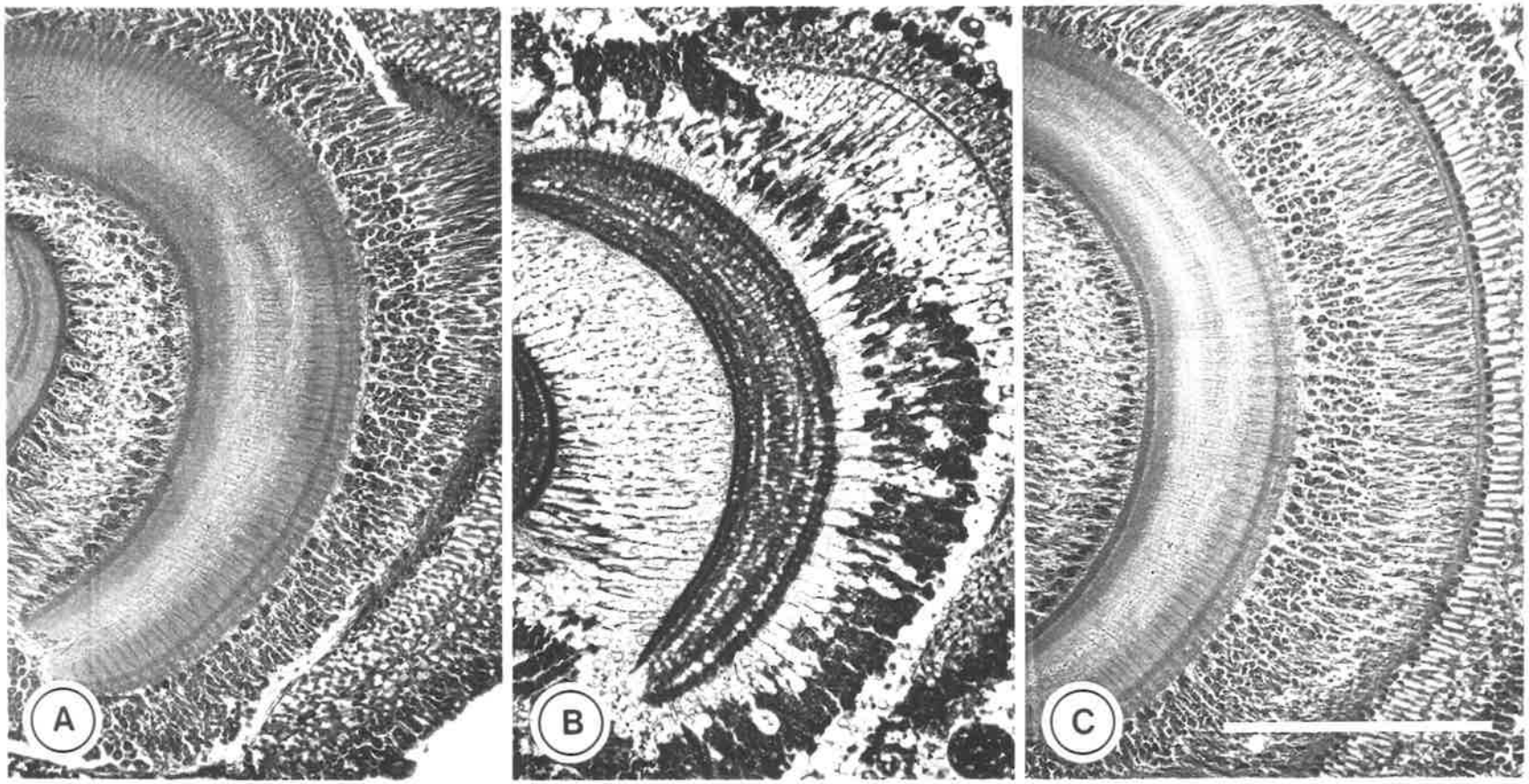

A
B
C

Figure 5.5

Morphology of cells of the optic lobe of an uninfected pupa (A), a KBV-infected pupa (B), and an SBV-infected pupa (C), each prepared 24 hr post-inoculation. The nature of KBV-induced damage to chiasmata cells - swelling and loss of cytoplasmic staining - is apparently very similar to that seen in glial cells (see Figure 5.4). Bouins fixative, Mallory's Triple Stain; each at identical magnification, bar = 250 µm.


A
B
C

detail, otherwise showing only that affected cells became rounded and enlarged. Electron microscopy was used for more extensive investigations of this response, and those results are given in Section 5.3.

The SBV-infected specimens illustrated in Figures 5.1 to 5.5 support the conclusion that at the light microscopy level there are no detectable pathological changes in SBV-infected cells. While these figures were chosen to display tissues most spectacularly affected by KBV, other tissues, in which studies revealed large amounts of SBV, also showed normal morphology.

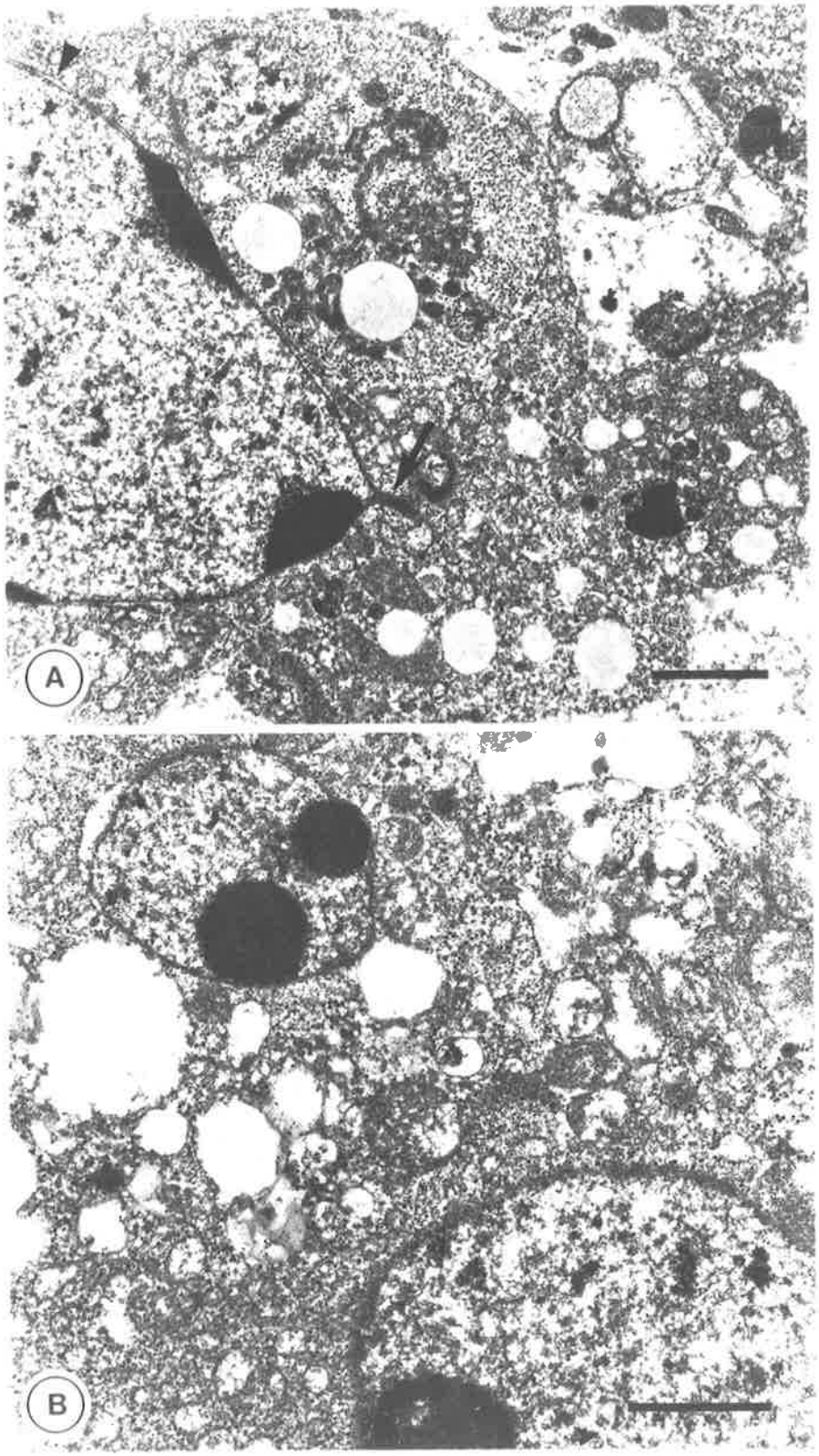
5.3 Electron microscopy studies

Examination of tissue specimens prepared for electron microscopy (EM; see Section 4.3.1 for methods) revealed the same distribution of KBV-induced damage as that detected by light microscopy. However, it also demonstrated the presence of pathological changes at a subcellular level, and enabled clarification of the more obvious changes already described (see Section 5.2).

Cells noted earlier as showing nuclear condensation and general rounding were found to display a variety of other pathological effects. Figure 5.6 shows representative parts of two such cells at early and terminal stages of KBV infection, which display moderate and extreme degrees of pathological damage, respectively. As shown in these micrographs, nuclear condensation was seen to result from progressive margination of the chromatin, in some cases accompanied by enlargement of the perinuclear space [Figure 5.6 (a), arrowhead], and invariably by deformation of the entire organelle, varying in degree from minor

Figure 5.6

KBV-infected foregut epithelial cells, prepared 40 hr post-inoculation. Micrograph (A) shows a cell at an early stage of infection; note minor deformation of nucleus (arrow), slight enlargement of perinuclear space (arrowhead) and necrotic and lobular appearance of cytoplasm. Micrograph (B) shows a cell at a later stage of infection; note condensation of chromatin in the two lobes of nucleus, enlargement of the perinuclear space around the nuclear lobe at top left, and large numbers of vesicles and vacuoles in the cytoplasm. In each micrograph, bar = 1 μm.


A
B

[Figure 5.6 (a), arrow] to severe, as suggested by the two lobes of nucleus visible in Figure 5.6 (b).

Pathological changes were also obvious in the cytoplasm of KBV-infected cells, and included the presence of those structures thought to be directly involved in viral replication, already discussed at length in Chapter 4. Other changes involved disintegration of organelles, the formation of unstructured granular inclusions and major vacuolation of the ground cytoplasm. Interestingly, examination at this level showed clear evidence of formation of lobules of cytoplasm, as noted previously in oenocytes (see 5.2.2). Oenocytes examined by EM showed internal changes similar to those outlined above, suggesting that all cells infected by KBV react in a similar manner.

As stated in Section 4.3.4, tissue of the nervous system was not infected by KBV, however, use of EM revealed evidence of another type of cellular response induced by the presence of the virus, and confined to glial and chiasmata cells. Figure 5.7 (a) to (c) show examples of this type of response in glial cells, arranged in a putative chronological sequence.

The initial response of glial cells involves formation in the cytoplasm of both complex membranous structures [Figure 5.7 (a), arrow], and large numbers of membrane-bound vacuoles. These vacuoles swell and coalesce to form large structureless, membrane-limited areas within the cell [Figure 5.7 (b)], and in doing so apparently isolate the cell nucleus from the compacted remnants of cytoplasm and cytoplasmic organelles. This results in progressive disintegration of both the nucleus, as shown in the sequence Fig. 5.7(a)-(c), and the membrane-

Figure 5.7

Effect of KBV infection on pupal nervous tissue. Micrographs (A)-(C) show glial cells in a putative sequence of disintegration, each prepared 40 hr post-inoculation. The progressive reduction in density of nuclear contents and integrity of nuclear membrane is accompanied by the formation in the cytoplasm of complex membranous structures [(A), arrow] and numerous vacuoles. The vacuoles appear to coalesce, forming membrane-limited areas within which cytoplasm becomes isolated, and finally breaks down [(B) and (C), arrows]. In micrographs (A)-(C), bar = 2 μm. Micrograph (D) shows the result of a similar process in a chiasmata cell from the optic lobe, prepared 40 hr post-inoculation. Bar = 4 μm.

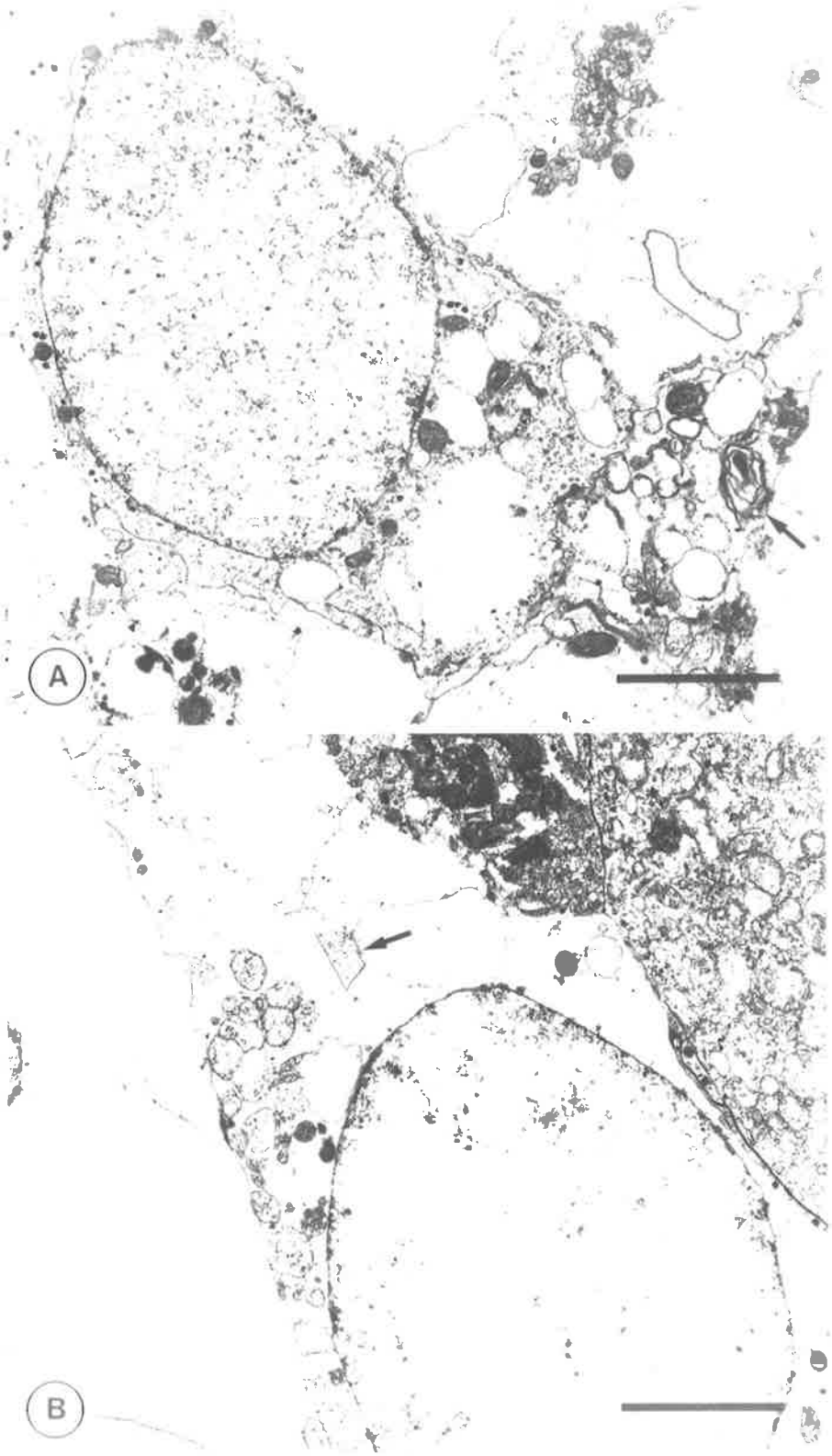
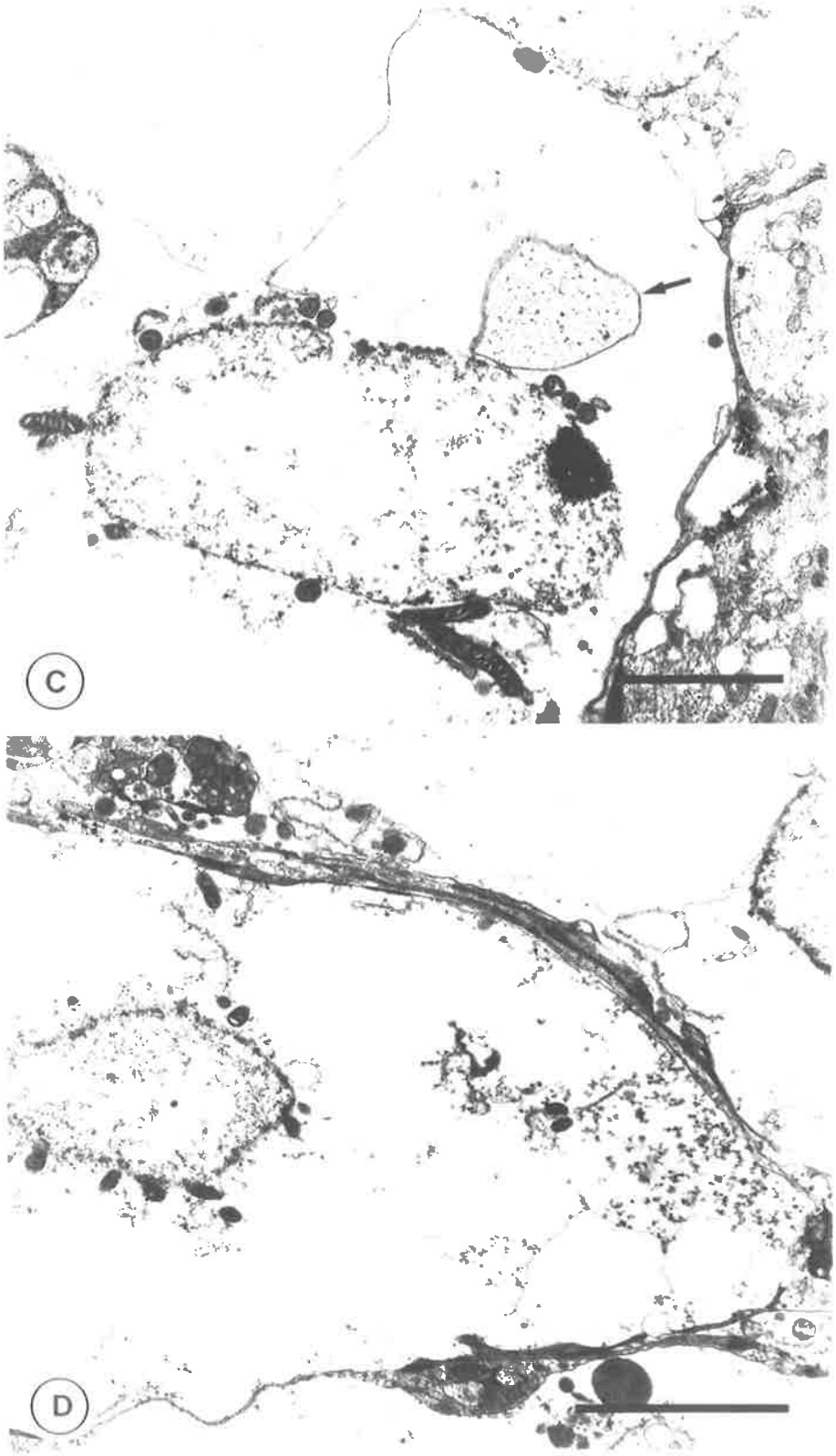

A
B

Figure 5.7

(continued; see previous page for explanation)


C
D

limited cytoplasm [Figure 5.7 (b) and (c), arrows], and leads to eventual formation of the large necrotic regions visible by light microscopy. A similar process appears to take place in the chiasmata cells of the optic lobe, one of which is illustrated in Figure 5.7 (d), in a terminal stage of the sequence.

Examination of cells infected with SBV failed to show changes of a pathological nature, with the exception of the cytoplasmic inclusion of replicative structures, as described earlier. As an example, Figure 5.8 shows an SBV-infected, but structurally normal cell from the hypopharyngeal gland of an infected pupa.

The results from Sections 5.2 and 5.3 have been summarised in Table 5.1, in a format directly comparable with that of Table 4.6.

5.4 Studies of KBV-induced damage to the pupal nervous system

5.4.1 Materials and methods

Measurement of haemolymph osmolality. A 10 µl haemolymph sample was withdrawn from each pupa into a silicon-coated microcapillary tube, through an incision made in the dorsal surface of the thorax. Samples were stored at -80°C, and osmolality was measured in the manner described (Section 3.2.1).

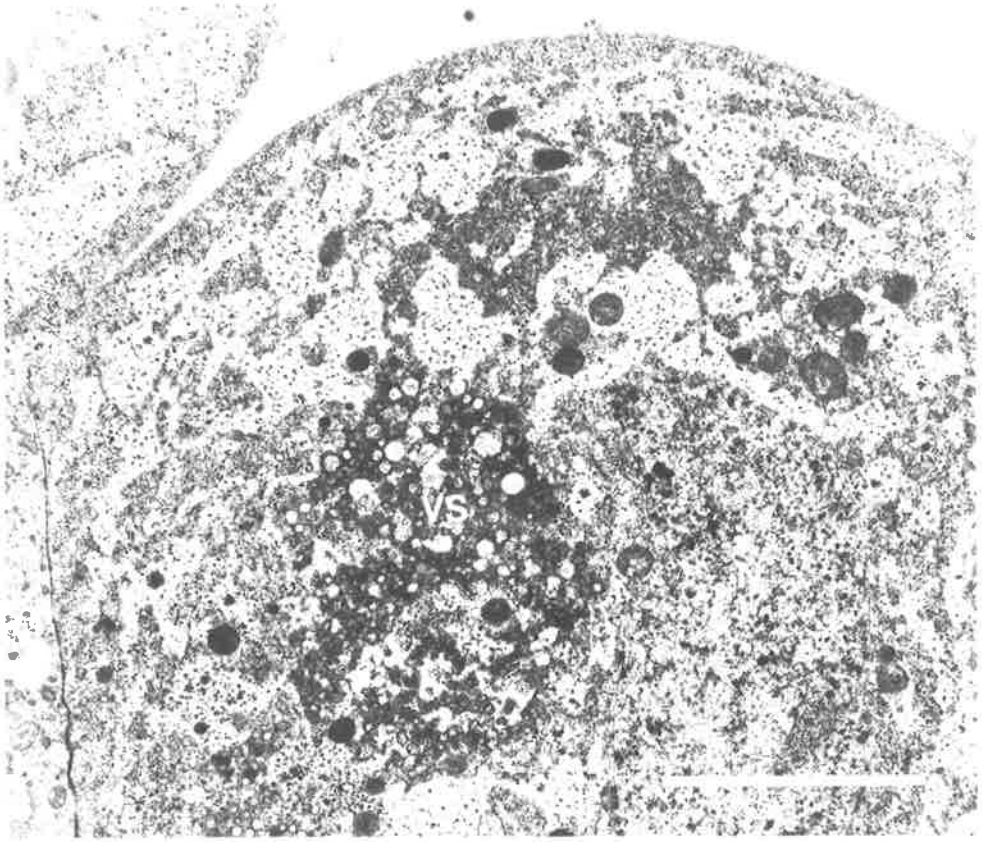
Preparation of virus-free inocula from concentrates of pupae. Attempts were made to concentrate a substance with neuropathic properties from homogenates of healthy and Kashmir virus-infected pupae.

Table 5.1 : Summary of tissue damage following infection of honey bee pupae with SBV or KBV, based on results described in Sections 5.2 and 5.3.

Tissue	SBV	KBV
Foregut epithelium	-	+
Midgut epithelium	-	-
Hindgut epithelium	-	+
Alimentary glands	-	-
Muscle	-	+
Fat body	-	-
Haemocytes	-	+
Oenocytes	-	+
Epidermis	-	+
Tracheal epithelia	-	+
Nervous system	-	+

Figure 5.8

Part of an SBV-infected hypopharyngeal gland cell, prepared 72 hr post-inoculation. Note the typical SBV-induced vesicular structure (VS) lying close to the cell nucleus (N). No virally-induced pathological changes could be detected in SBV-infected cells. Bar = 4 µm.


VS

Pupae were inoculated with 10^{-5} ng of KBV, or with 2/3 IR alone, and after incubation of 40 hr a group of 30 pupae was taken from each treatment. Each group was homogenised separately in 2 ml of ice-cold potassium phosphate buffer (5 mM, pH 7.0) and the homogenates were centrifuged at 7,500 *g* for 20 min. Three ml of each supernatant were then dialysed for 6 hr at 4°C against 240 ml of the trituration buffer. Each dialysate was dried by rotary evaporation at 32°C in a silicon-coated flask, and the resulting amorphous pale yellow deposit was resuspended in 1 ml of distilled water.

Measurements were made of the UV spectra of 1% solutions of the samples. The sample derived from KBV-infected pupae had an $A_{260/280}$ ratio of 1.29, and a maximum absorbance of 0.896 at 250 nm wavelength, while that from healthy pupae had a ratio of 1.27, and a maximum of 0.733 at 259 nm.

Two experiments were conducted with these extracts. For the first, three series of inocula were made by successive dilution of aliquots of each of the resuspended extracts, and of a control potassium phosphate buffer solution of similar strength (1.2 M, pH 7.0). Sterile 2/3 IR was used as dilutant, and inocula ranged in strength from undiluted to a dilution of 1/20. Each pupa received a 3 µl dose of one inoculum, was incubated for 18 hr, and then dissected. Heads and abdoma were prepared for light microscopy as described (Section 5.2.1), and thoraces were homogenised and tested by double immunodiffusion to ensure that pupae were free from KBV infection. The results of this experiment, reported in Section 5.4.3, determined the design of the experiment which followed.

The second experiment used the same methods as the first, but because that experiment had shown no activity in inocula prepared from control pupae or phosphate buffer, the second tested only the pupal extracts. For the second experiment both extracts were diluted to give an approximately equal A_{280} reading (4.47 for KBV extract and 4.43 for that from healthy pupae). Abdoma only were prepared for microscopy, while heads and thoraces were used to test for KBV infection.

5.4.2 The role of haemolymph osmolality

Assuming that an indirect mechanism of damage must be mediated through the haemolymph, and knowing the effect of picornavirus infection on cellular membrane permeability (see Section 5.5), it seemed pertinent to examine haemolymph osmolality for changes both associated with viral infection, and potentially capable of causing cytopathological damage.

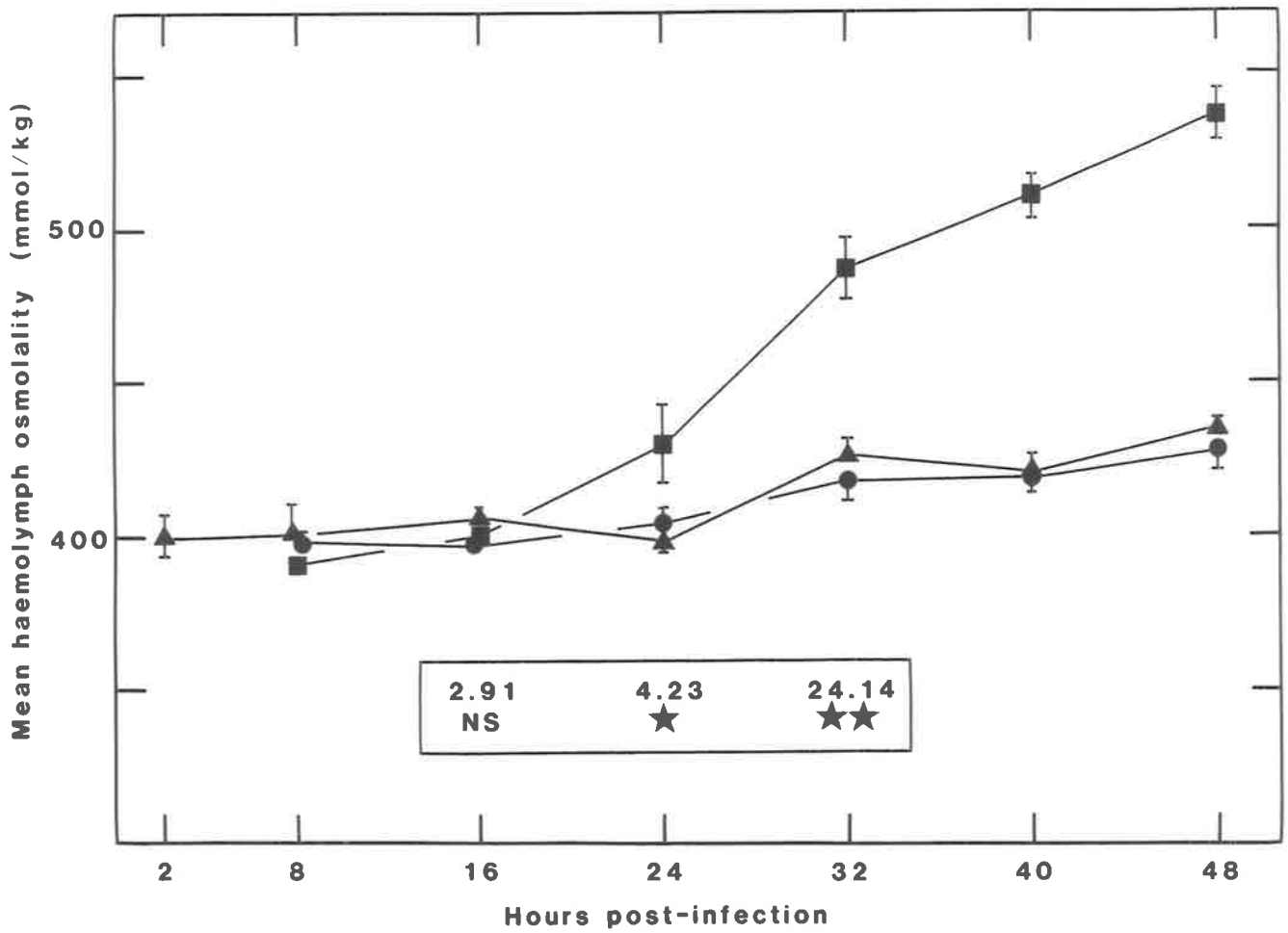
The data presented in Figure 5.9 show that SBV infection had no effect on haemolymph osmolality ; in contrast, within 24 hr of infection with KBV there was a significant rise in osmolality, from about 400 to 430 mmol/kg. Between 24 hr post-infection, and the death of the animal (see Section 4.2.4), haemolymph osmolality continued to increase at an approximately uniform rate.

Since glial cell damage was also found to occur within 24 hr of KBV infection, it was inferred that the minimum osmotic requirement for cell damage must have been 8 hr exposure to an osmotic potential of 430 mmol/kg.

Figure 5.9

Changes in haemolymph osmolality of control (2/3 IR-inoculated) and infected A. mellifera pupae. Plots show mean with SE of mean (n=5; error bars not shown when smaller than symbols); inset shows value and significance of F-statistic for indicated observation time.

▲ 2/3 IR; ● SBV; ■ KBV.


Mean haemolymph osmolality (mmol/kg)
500
400
2.91
NS
4.23
★
24.14
★★
2
8
16
24
32
40
48
Hours post-infection

In an attempt to mimic these haemolymph conditions, groups of pupae were inoculated with increasing concentrations of Insect Ringers solution. Each pupa received 3 µl of inoculum and was incubated for 18 hr, then a sample of haemolymph was collected as described (Section 5.4.1).

The data in Table 5.2 show that this method could successfully increase pupal haemolymph osmolality to the levels found in infected animals. However, no trace of damage was seen in brain tissue of treated pupae when examined by light microscopy (Figure 5.10).

5.4.3 The role of neurotoxic substances of pupal origin

Concentrated dialysates of extracts prepared from pupae were found to be highly toxic when inoculated into test pupae, causing a rapid melanisation reaction, initially at the site of inoculation, and later throughout the body. This response occurred after inoculation of test pupae with preparations derived from either KBV-infected or mock-infected pupae, but was not seen in test pupae inoculated with the control buffer.

Serial dilutions of the extracts caused proportionately less damage, so that following inoculation with 15-fold dilutions pupal survival rate was about 75%. Dilutions of this order were used in both experiments, since they appeared to represent a compromise between a reasonable survival rate, presumably with a minimum amount of non-specific damage, and a maximum exposure of test pupae to the putative toxin(s).

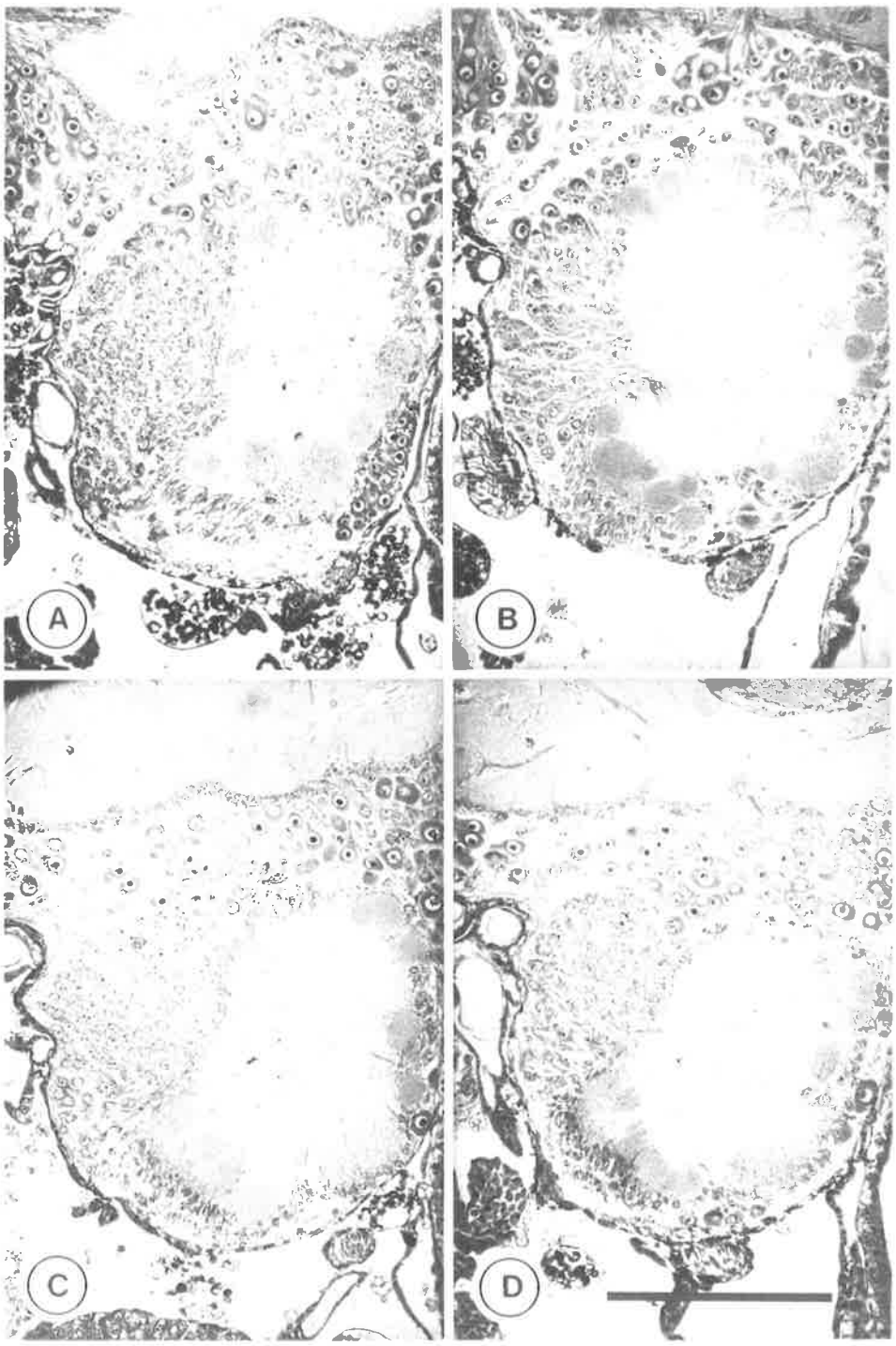
Table 5.2 : Pupal haemolymph osmolality after inoculation with concentrated salt solution.

Inoculum	Inoculum osmolality (mmol/kg)	Haemolymph osmolality (mmol/kg)
2/3 IR	205	405 ± 13#
IR	308	416 ± 14
2 x IR	615	468 ± 19
5 x IR	1538	451 ± 11
10 x IR	3075	463 ± 13
20 x IR	6150	572 ± 11

Mean osmolality ± S.E. of mean, sample size of 5.

Figure 5.10

Morphology of cells of the proto- and deutocerebrum following alteration of pupal haemolymph osmolality (see text for details). Specimens (A)-(D) were prepared from pupae which had haemolymph osmolalities of 405, 435, 454 and 465 mmol/kg, respectively; no morphological differences were detected between preparations from these pupae. Each at identical magnification; bar = 150 µm.


A
B
C
D

Serological tests showed that three of 56 pupae inoculated with dialysates of extracts subsequently became infected with KBV. These three pupae had all been inoculated with extract prepared from KBV-infected pupae and were not used in any further experimental procedure.

Examination of sections prepared for light microscopy from inoculated pupae showed a number of instances of pathological changes to nervous tissue (see Table 5.3). Although such changes were not seen in brain tissue they were apparent in all the examined sections of abdominal ganglia from affected individuals.

The histological appearance of extract-affected tissue was strongly reminiscent of the appearance of nervous tissue taken from pupae infected by KBV. While most cases of extract-induced damage were less severe, affected ganglia showed obvious swelling and heterogeneous staining of tissue elements, and in particular of glial cells (see Figure 5.11). Similar damage was visible in nerve trunks connecting abdominal ganglia, but cellular damage was not seen in any other tissue system.

Results from the first experiment (see Table 5.3) suggested that neuropathic activity was confined to the extract prepared from diseased pupae, but when more equal (based on total protein) concentrations of extracts were used, activity could be demonstrated in both preparations. No conclusive explanation can be given for the seemingly different levels of activity of the KBV extract in experiments 1 and 2, but plausible suggestions might include differential susceptibilities for pupae from different hives, and loss of dialysate activity during frozen storage in the rehydrated state.

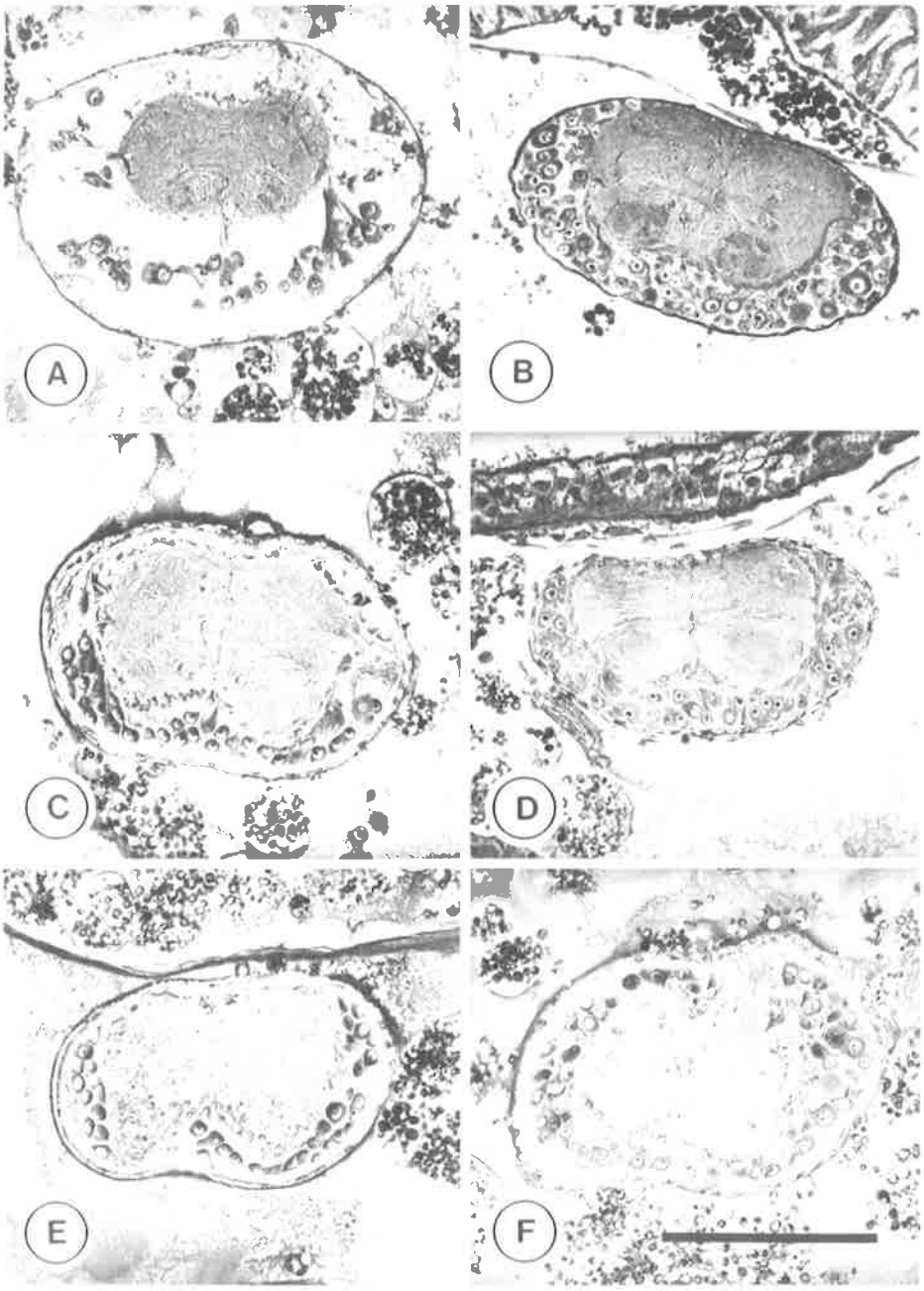
Table 5.3 : Effect of inoculation of dialysate preparations from healthy and KBV-infected pupae on cytopathology of nervous tissue.

Inoculum		Experiment 1		Experiment 2
Source	Reciprocal dilution	Head	Abdomen	Abdomen
KBV-infected pupae	15	0/2#	2/2	1/4
Healthy pupae	15	0/2	0/2	-
	13	-	-	2/4
Phosphate buffer	15	0/2	0/2	-

Numerator/denominator gives number of pupae showing pathological changes to nervous tissue/number of pupae examined.

Figure 5.11

Morphology of abdominal ganglia following attempts to mimic KBV-induced cellular damage (see text for details). (A) and (B) show sections of abdominal ganglia from KBV-infected and uninfected pupae, respectively. Others show sections of ganglia from pupae inoculated with dialysate preparations from KBV-infected (C and E) or healthy pupae (D and F) in either experiment 1 (C and D) or 2(E and F). (C), (E) and (F) show swelling of glial cells in a manner less severe, but reminiscent, of changes induced by KBV infection. Each at identical magnification; bar = 150 µm.


A
B
C
D
E
F

5.5 Discussion

Cells infected by SBV showed no changes of a pathological nature, except for the inclusion of virally-associated structures and particles. This finding is in agreement with general statements by Bailey and Fernando (1972) that SBV infection does not cause obvious symptoms in adults, and largely in agreement with the results of Mussen and Furgala (1977). These latter authors did, however, note occasional damage to hypopharyngeal gland cells ; this apparent discrepancy could plausibly be explained as resulting from differences in the physiology or susceptibility of cells from the maturing, inactive glands of pupae, compared to those of the fully functional glands of adult workers.

Symptomless picornaviral infection of vertebrates is frequently associated with host seroconversion, and in such cases there is often a tacit assumption that infection has been limited by the host immune response. If this was always so, a different mechanism would be required to explain the limitation of such infections in insects, since the invertebrate immune system, while not fully understood (Warr, 1981) seems unlikely to possess such capabilities.

It seems, however, that different members of the Picornaviridae show wide variation in their cytopathic effects, with some viruses causing little or no damage to infected cells, regardless of the host immune response. Thus, for example, hepatitis A virus (HAV) has been shown to cause persistent, but inapparent, infections in foetal monkey kidney cells (Flehmig, 1981) and embryonic human fibroblast cells (Vallbracht et al., 1984), even though, in the latter case at least,

such cells were not protected by either specific antibodies or interferon.

The apparent balance between multiplication of SBV and the metabolism of the pupal host cell is similar in many, but not all, respects to the infections established by HAV. Thus although HAV has never been shown to cause direct cytopathological damage (Vallbracht et al., 1984), the outcome of SBV infection seems to be heavily influenced by host cell physiology, as illustrated by the obvious difference in status of larval, and pupal and adult infections.

In marked contrast to the 'balanced' infections described above are those cases in which viral infection causes major cytopathic effects (CPE). In most infected cells CPE are associated with a common spectrum of symptoms, such as swelling and rounding, and alterations in nuclear shape and texture. On occasion other more distinctive features, such as nuclear or cytoplasmic inclusion bodies, may be present, although these are not commonly associated with picornaviral infection (Luria et al., 1978).

In the present study, cells infected with KBV were found to demonstrate most of these responses, but did not display unique or remarkable symptoms by which this virus could be allied or differentiated from other small RNA viruses.

Probably the most interesting aspect of this study was the discovery of KBV-induced indirect damage to glial cells of the host nervous system.

Initially it was thought that this might result from the increased haemolymph osmolality observed in KBV-infected hosts, but experimental manipulation of this parameter failed to demonstrate any association. The basis of the observed change in osmolality following infection by KBV is not certain, but may be due to movement of cytoplasmic materials through 'leaky' membranes of infected cells (see Pasternak and Micklem, 1981), to cytolytic liberation of cellular contents into the haemocoel, or through a combination of both.

Damage of a type similar to that seen in early stages of KBV infection was, however, induced by inoculation of pupae with concentrated extracts prepared from other pupae. This finding suggested that such damage was normally caused by release of toxic material during lysis of necrotic cells, a phenomenon known to be involved in tissue irritation in other types of infectious disease (see Mims, 1982).

Experimentally-induced disruption of glial cell structure was confined to ganglia close to the site of inoculation, so that following abdominal injection, abdominal ganglia, but not brain tissue, were affected. This observation is not thought to invalidate the explanation given above, since necrotic cells of an infected animal would liberate such toxins continuously, and in all parts of the body, from sites of viral tissue damage (see Sections 5.2 and 5.3).

While no information can be provided about the mode of action of the neurotoxic substance (or substances), its movement through dialysis membrane suggests that it is less than about 2×10^4 daltons in size (supporting data not shown). Although such a size would not disqualify a number of hydrolytic enzymes, the apparently unique site of action

suggests that a more specific mechanism might be involved. It is possible, for example, that substances with specific toxic activity might be released by virally-induced (or experimental) disruption of subcellular organelles, such as lysosomes. Such neuro-specific toxicity has recently been demonstrated by a synthetic compound, methylphenyltetrahydropyridine (MPTP), which causes selective damage to cells of the substantia nigra region in a number of vertebrates, producing manifestations similar to those of Parkinson's disease (Langston et al., 1983).

Finally, it should be noted here that the extract inoculation procedure bears certain similarities to that described in Section 3.3, where it was used to induce inapparent infections to multiply to detectable levels. Because of the risk of KBV contamination from activated inapparent infections, or from the toxin preparation itself, heads and/or thoraces of all inoculated pupae were tested serologically. Infections were observed in only three of 56 pupae, but since all of these had been inoculated with the extract prepared from KBV-infected pupae, the source of the infective virus could not be determined. All of the pupae used in the histological examinations gave negative results by this test.

CHAPTER 6 : MIXED INFECTIONS OF SACBROOD AND KASHMIR VIRUSES

6.1 Introduction

Simultaneous infection of an individual host by more than one pathogen can frequently produce variation in both symptom expression and pathogen replication. Kurstak et al. (1974) considered that multiple viral infection could lead to qualitative (phenotypic and/or genotypic) or quantitative changes in replication, or alternatively, to the independent multiplication of each. More recently it has also been shown that multiple infections can cause changes to the pattern of viral distribution within host tissues (Carr and Kim, 1983). In situations where an interaction occurs, the outcome of a mixed infection has generally been found to depend on the multiplicity of each infecting virus, and on the delay between infection and superinfection.

Such interactions frequently result in reduced yields of one or both of the viruses, a phenomenon known as viral 'interference', and defined by Fenner et al. (1974) as a condition in which infection with one virus leads to 'resistence of cells or tissues to infection by a challenge virus'.

Both independent and interactive virus multiplication have been demonstrated in a variety of insect hosts, generally with large or easily distinguishable pathogens such as iridoviruses or baculoviruses, or with the mosquito-infecting toga- or bunyaviruses (see, for example, Tanada, 1971 ; Kurstak and Garzon, 1975 ; Davey et al., 1979). Similar results have been obtained by using these viruses to infect insect cell lines (for example, Dittmar et al., 1982).

Very few studies, however, have used small RNA viruses of insects. Friesen et al. (1980) reported homotypic interference between maturation-defective and wild-type black beetle virus (BBV) during replication in cultured Drosophila cells, while Scotti (in Scotti et al., 1981) found that growth of cricket paralysis virus in these cells was apparently unaffected by the presence of the defective virus.

Little work has been done on the consequences of multiple viral infection in honey bees, even though individuals collected from a single hive frequently contain a number of different isolates. The only quantitative study appears to be that of Bailey and Milne (1969), who could find no specific interaction between acute and chronic paralysis viruses replicating in adult workers.

The experiments reported here were originally suggested by preliminary results from the study of inapparent viral infections, as described in Chapter 3. Those results seemed to imply that inoculation of groups of pupae with immune rabbit serum caused an increase in the number of detectable heterologous infections within that group. While this increase was later shown to be circumstantial, it led to the hypothesis of specific interaction between SBV and KBV, an idea supported by the data which follow.

6.2 Establishment of double infections

In Table 6.1, data have been arranged to show the establishment of single and mixed infections following the injection of different combinations of inocula. The ability of these inocula to initiate single

Table 6.1 : Effect of relative viral multiplicity and the delay between first and second inoculation on establishment of infection.

Treatment	Inocula First ; Second	Resultant Infection	Number of pupae which developed infection when the second inoculation was delayed by the period shown below.# 0*	2-3 hr	5-7 hr	10 hr	20 hr	38-42 hr	
1	SBV, 10^{-3} ng ; KBV, 10^{-5} ng	SBV only	0	0	0	0	0	0	1
		SBV + KBV	0	0	18	20	19	24	19
		KBV only	20	24	2	0	1	0	0
		(Total inoculated)	(20)	(24)	(20)	(20)	(20)	(24)	(20)
2	SBV, 10^{-1} ng ; KBV, 10^{-5} ng	SBV only	0	0	0				0
		SBV + KBV	17	20	24				24
		KBV only	3	0	0				0
		(Total inoculated)	(20)	(20)	(24)				(24)
3	SBV, 10^{-3} ng ; KBV, 10^{-7} ng	SBV only	15	20					14
		SBV + KBV	4	2					10
		KBV only	1	2					0
		(Total inoculated)	(20)	(24)					(24)
4	SBV, 10^{-1} ng ; KBV, 10^{-7} ng	SBV only	6		21				6
		SBV + KBV	14		3				17
		KBV only	0		0				0
		(Total inoculated)	(20)		(24)				(23)

* Simultaneous inoculation
\# Presence of virus detected by ELISA (Section 2.6)

infections has been discussed in Section 4.2.1, and those results and the data presented here show a good general agreement.

The data presented in Table 6.1 show that two factors - the concentration of virus in the other inoculum, and the length of delay between primary and secondary inoculation - both exercise a significant influence on the establishment of double infections. When, for example, pupae were inoculated with an ID_{100} (see 4.2.1) of both SBV and KBV (Table 6.1, treatment 1), the delay between the two inoculations was of critical importance ; it was found, however, that the influence of this parameter could be minimised by substantially increasing the concentration of the SBV inoculum (Table 6.1, treatment 2). A similar interdependence of virus concentration and inoculation delay was detected in all combinations assessed in Table 6.1, although greater variability in establishment of infection made interpretation of results more difficult when assessing combinations which used very low inoculum levels.

While the results presented in this section are essentially qualitative, the next two sections report quantitative investigations of the influence of these factors on viral multiplication.

6.3 Influence of delay between primary and secondary inoculation on virus growth

This section contains results from two experiments, each designed to test a different aspect of the influence of superinfection delay on virus multiplication. In both, pupae were inoculated with an ID_{100} of each virus (10^{-3} and 10^{-5} ng/pupa for SBV and KBV, respectively), with

length of elapsed time between inoculations being the only variable. The data presented here and in Section 6.4 have been analysed by non-parametric methods, since initial testing showed widespread variance non-homogeneity, thus precluding the use of a parametric approach.

Figure 6.1 shows the yields of each virus produced from mixed infections, and the manner in which these varied as superinfection delay was increased. Table 6.3 contains further information about the rates of virus growth under these circumstances. Despite some minor differences, the results of these experiments show good general agreement.

In both experiments (Fig. 6.1 and Table 6.3), simultaneous inoculation of the viruses resulted in failure of SBV to establish infection, as discussed in Section 6.2. In one experiment (Figure 6.1 and Table 6.2b) simultaneous inoculation did not affect the final yield of KBV, compared to that from single infected pupae, while in the other (Table 6.3) it was accompanied by about a 25% decrease in yield, which was statistically significant ($p < 0.001$).

It is clear from Figure 6.1 that increasing the delay between primary inoculation (with SBV) and secondary inoculation (with KBV) resulted in multiplication of the former being favoured at the expense of the latter. As shown in Table 6.2, every increase in superinfection delay resulted in significant increase in the final yield of SBV (as compared to those from shorter delays), until 40 hr, when the same yields were recovered from both single- and double-infected pupae.

Figure 6.1

Quantity of each virus recovered from individually tested double-inoculated pupae. Inoculation of KBV (10^{-5} ng/pupa) followed that of SBV (10^{-3} ng/pupa) by the period shown; pupae were incubated for 96 hr plus length of delay. Yields from sibling pupae infected with only one virus, and incubated for 96 hr, are shown for comparison. Plots show mean (µg) with SE of mean, n = 10. ● SBV; ■ KBV.

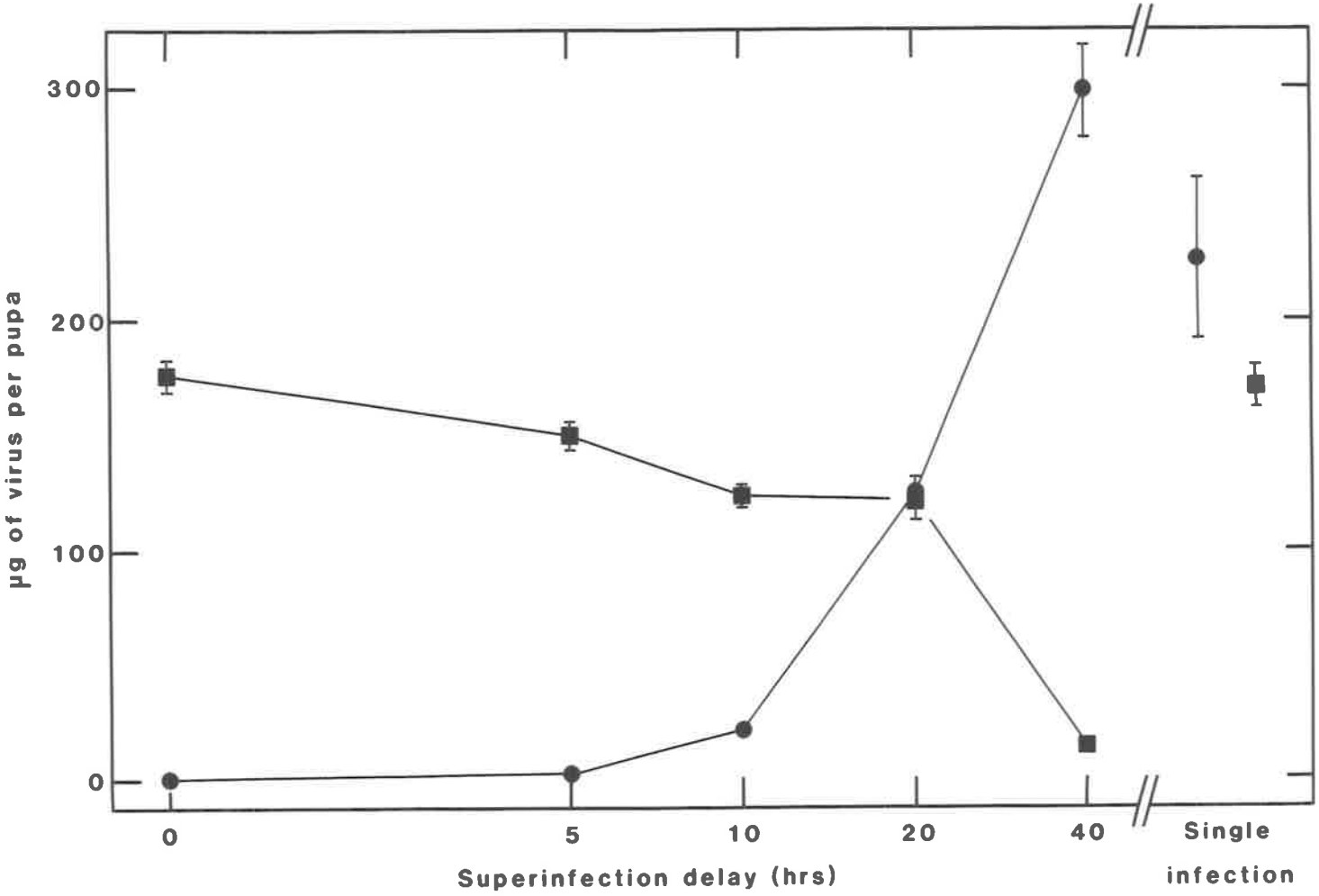

300
200
100
0
µg of virus per pupa
0
5
10
20
40
Single infection
Superinfection delay (hrs)

Table 6.2 : Kruskal-Wallis and Mann-Whitney statistics, with significance levels, for data from Figure 6.1.

(a) Comparison of SBV yields from single and double infections.

Kruskal-Wallis test. H_o : no significant differences between yields of SBV. H-statistic (adjusted for ties) = 73.88 ; $p < 0.001$ therefore reject H_o.

Mann-Whitney tests : W-statistic (significance).

		Delay (hrs)				
	0+	5	10	20	40	SBV alone
Delay (hrs)	5	≠	172 (***)	171 (***)	171 (***)	171 (***)
	10		.	190 (***)	190 (***)	190 (***)
	20			.	202 (***)	194 (***)
	40				.	277 (N.S.)

\+ cannot compare result from simultaneous inoculation

≠ not applicable

(Table 6.2, continued)

(b) Comparison of KBV yields from single and double infections.

Kruskal-Wallis test. H_o : no significant differences between yields of KBV. H-statistic (adjusted for ties) = 74.89 ; $p < 0.001$ therefore reject H_o.

Mann-Whitney tests : W-statistic (significance).

		Delay (hrs)					
		0_+	5	10	20	40	KBV alone
	0_+	≠	484	577	551	590	450
			(**)	(***)	(***)	(***)	(N.S.)
	5		.	459	426	513	294
Delay (hrs)				(**)	(*)	(***)	(N.S.)
	10			.	394	590	254
					(N.S.)	(***)	(***)
	20				.	551	254
						(***)	(***)
	40					.	190
							(***)

\+ simultaneous inoculation

≠ not applicable

Table 6.3 : Quantity of each virus recovered from individually-tested pupae after growth as single or mixed infections for time shown.

Inocula First : Second (dose/pupa)	Delay (hrs) between infection & super-infection	Virus	Quantities of virus (µg) per pupa — Period of viral replication (hrs)* 20	40	60	80
SBV 10^{-3} ng : -	-	SBV	3 ± 0#	143 ± 2	NDø	608 ± 46
KBV 10^{-5} ng : -	-	KBV	136 ± 7	142 ± 7	ND	132 ± 9
SBV 10^{-3} ng : KBV 10^{-5} ng	0+	SBV	0§	0	ND	0
		KBV	106 ± 3	106 ± 6	ND	100 ± 4
SBV 10^{-3} ng : KBV 10^{-5} ng	20	SBV		114 ± 9	218 ± 19	245 ± 15
		KBV		59 ± 9	136 ± 6	128 ± 6
SBV 10^{-3} ng : KBV 10^{-5} ng	40	SBV			434 ± 30	562 ± 46
		KBV			7 ± 1	52 ± 6

* Period of virus growth is quoted as time since first inoculation.
\+ Simultaneous inoculation.
\# Mean (µg) ± S.E. of mean, sample size of 10.
§ Below detection threshold.
ø Not determined.

In contrast, delays of 10 hr or greater led to significant reductions in KBV yield, as compared to those from single infections (see Table 6.2), and in all but one instance (10 hr vs. 20 hr delay), longer delays produced progressively smaller yields.

Data in Table 6.3 show that these reductions in yield were associated with changes in the rates of growth of both viruses. Thus, for example, when KBV was inoculated simultaneously with SBV, or was grown alone, its multiplication was completed within a 20 hr period, but when inoculated 20 or 40 hr after SBV, its period of multiplication was greatly prolonged. These data also show that the subsequent KBV superinfection could cause a marked decline in the rate of growth of SBV.

These alterations in growth rates suggest that the results reported here do not simply reflect a variation in time of host death, but instead represent a genuine example of viral interference. This point is discussed in greater detail in Section 6.6.

6.4 Influence of relative multiplicity on virus growth

In the previous section it was shown that the outcome of a mixed infection of SBV and KBV could be greatly influenced by the length of the delay before superinfection. That finding was, however, derived from experiments which used only one combination of inocula (an ID_{100} of each virus). This section contains data which demonstrate viral interaction after infection of pupae with other combinations of inocula, and which confirm that the earlier results reflect the existence of a more extensive phenomenon.

In the experiment described here (see Table 6.4), a constant delay of 40 hr was allowed between the primary (SBV) and secondary (KBV) inoculation. A delay of this length was earlier shown to be associated with significant reduction in KBV growth (Figure 6.1 and Table 6.3), and with more or less unaltered levels of SBV replication.

Table 6.4 reports final yields of each virus recovered from pupae in which mixed infections had been initiated with one of four different inoculum combinations. As shown, these results were essentially consistent with those of the experiments described in Section 6.3.

When grown as a single infection, an SBV inoculum of 10^{-1} ng/pupa was found to produce a significantly ($p < 0.001$) greater yield of virus than an inoculum of 10^{-3} ng/pupa, and had also produced significantly ($p< 0.001$) more virus (152 ± 19 vs. 42 ± 3 µg/pupa) at the time of KBV inoculation.

In three of the four inoculum combinations, SBV replication was not affected by the introduction of KBV, as judged by comparison of final yields from single and mixed infections. In the other case (SBV, 10^{-1} ng/pupa with KBV, 10^{-5} ng/pupa) there was a marked reduction in SBV yield, to a level which did not differ significantly from that produced by an SBV inoculum of 10^{-3} ng/pupa.

Like SBV, as a single infection the more concentrated inoculum of KBV (10^{-5} ng/pupa) produced a significantly ($p < 0.05$) greater amount of virus than the smaller (10^{-7} ng/pupa). In every case, however, the presence of SBV reduced multiplication of KBV to such an extent that no difference could be detected between final yields from either inoculum.

Table 6.4 : Quantities of each virus recovered from individually-tested pupae after infection with various combinations of inocula.*

First inoculum (dose/pupa)	Quantities of virus (µg) per pupa						
	Second inoculum (dose/pupa)						
	KBV, 10^{-5} ng		KBV, 10^{-7} ng		2/3 IR	Uninjected	
	SBV	KBV	SBV	KBV	SBV	SBV	KBV
SBV, 10^{-1} ng	133 ± 13§ (24)	62 ± 8 (24)	234 ± 22 (17)	59 ± 8 (17)	192 ± 23 (7)	262 ± 22 (30)	-†
SBV, 10^{-3} ng	122 ± 11 (23)	60 ± 4 (23)	148 ± 14 (10)	44 ± 4 (10)	107 ± 20 (10)	117 ± 9 (30)	-
Uninjected+ or 2/3 IR#	-	226 ± 14+ (30)	-	158 ± 23+ (13)	-	0# (19)	0# (19)

* Double-infected pupae were incubated for a total (i.e. after first infection) of 112 or 160 hr, single-infected for 80 to 160 hr. All data were tested for homogeneity before being pooled to give tabulated values.
§ Mean yield (µg) ± S.E. of mean, (number in sample).
† Not applicable.

It should be noted that a second inoculation of SBV-infected pupae with 2/3 IR had no significant effect on the final SBV yield, suggesting that secondary 'wounding' did not disturb host physiology to an extent sufficient to cause a detectable reduction in viral multiplication.

These results indicate that the viral interaction described in this chapter persists across the range of inoculum combinations likely to be of importance under field conditions.

6.5 Histopathology of mixed infections

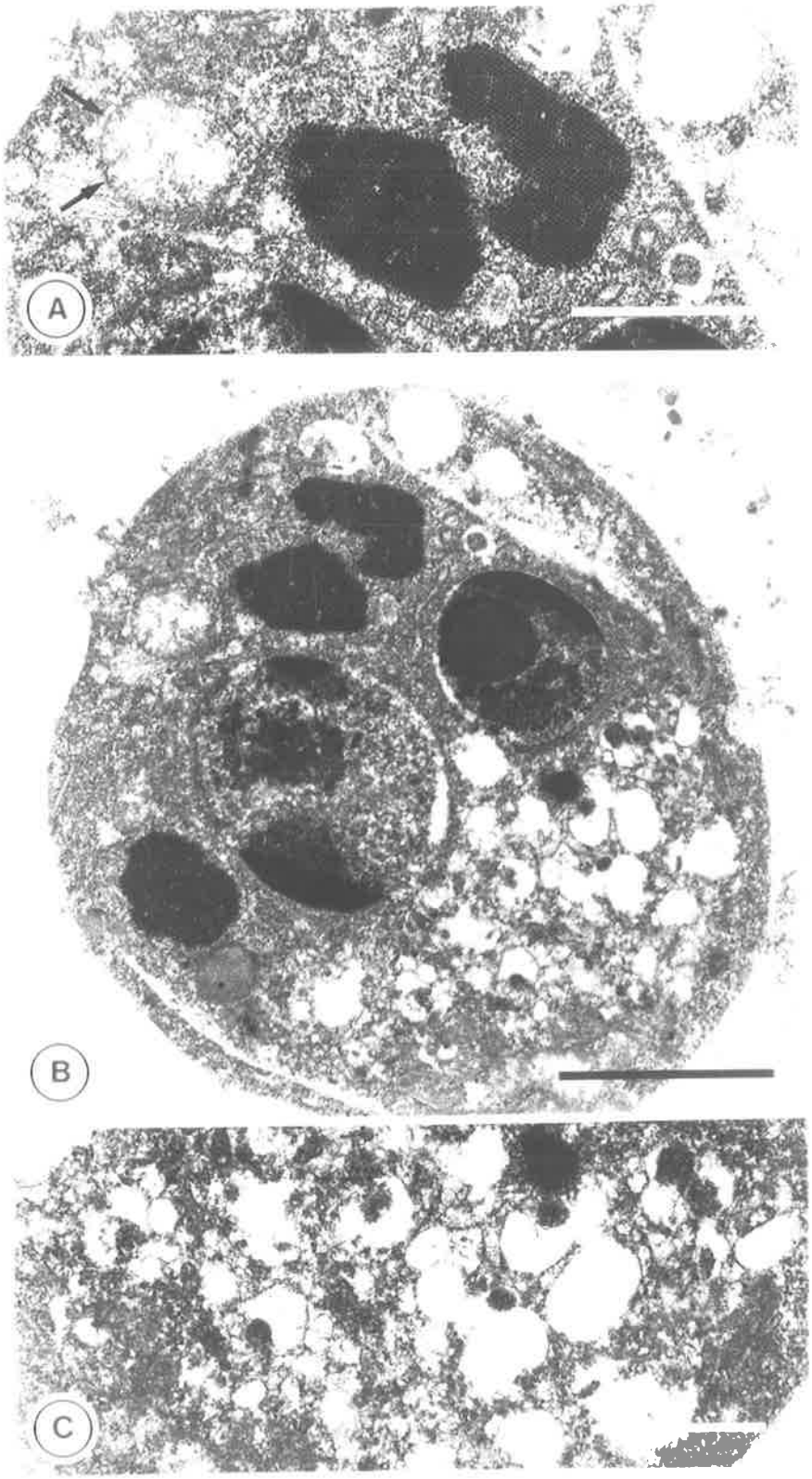
On the basis of susceptibility to infection, pupal tissues may be categorised as permissive for replication of one, both, or neither of the two viruses (see Table 4.6). The results presented in this section describe electron microscopical observations of preparations from doubly-infected pupae, and more precisely, of tissues from two of these categories - the foregut and the central nervous system.

Foregut tissue has previously been determined as being capable of supporting growth of single infections of each virus. In doubly-infected pupae both viruses were found in this tissue, and in some rare cases each was apparently present in the same cell, as shown in Figure 6.2, and described below.

Cells of the foregut appeared to respond to double infection in one of two distinct ways. One type of response (see Figure 6.2) was similar to that induced by single KBV infections, resulting in such effects as cellular rounding, condensation of chromatin, and destruction of cytoplasmic organelles. In many cases vesicular structures, apparently

Figure 6.2

Foregut epithelial cell from a double-infected pupa (SBV with KBV, 72 and 48 hr post-inoculation, respectively). Micrograph (B) shows a low magnification view of the entire cell which, in general appearance, resembles those infected by KBV (see Figure 5.6). Micrographs (A) and (C) show areas of (B) at higher magnification. (A) shows a membranous vesicle similar in type to those associated with KBV replication (arrows; see also Figure 4.11), while (C) shows part of a vesicular structure typical of those induced by SBV infection (see Figure 4.8). Bar = 1 µm (A) and (C); 2 µm (B).


A
B
C

identical to those induced by single SBV infections, were present in the cytoplasm of these cells [see Figure 6.2 (c)], and occasionally the inclusion bodies characteristic of SBV replication were also visible. Conclusive evidence of KBV replication was usually more difficult to find ; Figure 6.2 (a) shows a cytoplasmic inclusion (arrows), which, on the basis of size, contents and membrane-bound nature is thought to be a vesicle of the type known to be associated with KBV replication. Large numbers of viral particles were often present in the cytoplasm of these cells, and were seen to be distributed both randomly and in aggregations ; the pattern of distribution, however, was not regarded as being diagnostic for the presence of one or other of the viruses.

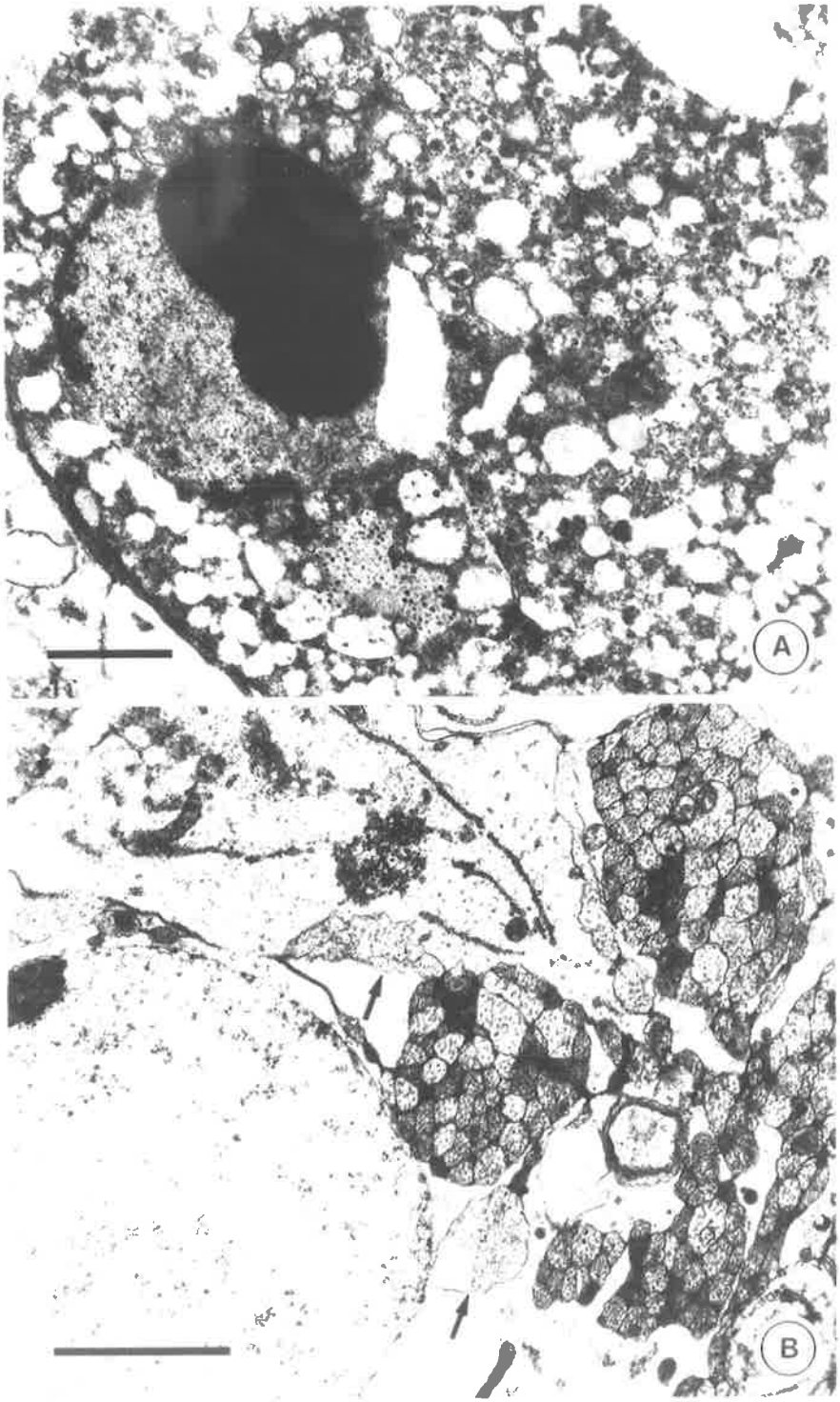
The other type of cellular response, illustrated in Figure 6.3 (a), appears to differ qualitatively from that seen in either type of single infection. Although the cell shown in this figure is somewhat rounded, irregular cellular distortions were commonly observed. This response included change of the cytoplasm to a 'granular' form, extensive condensation of chromatin, and additionally, formation of numerous membrane-bound vesicles and large numbers of apparently unbound cytoplasmic vacuoles. It is not known whether this vacuolation of the cell is a consequence of cytoplasmic contraction during 'granulation', or whether it is associated with some entirely different phenomenon, such as a generalised necrotic response. There was little indication that either vacuoles or vesicles were associated with viral replication, and figures such as the vesicular structures, described earlier, were not seen.

Mixed infections induced pathological changes in nervous tissue similar to those described from single KBV infections (see Section

Figure 6.3

(A) Foregut epithelial cell from a double-infected pupa (SBV with KBV, 72 and 48 hr post-inoculation, respectively). Note 'granular' appearance of cytoplasm, and presence of numerous vacuoles and membrane-bound vesicles; virus replication was not detected in such cells. Bar = 2 µm.

(B) Glial cell from a double-infected pupa (SBV with KBV, 72 and 48 hr post-inoculation, respectively). Some glial investment remains around bundles of axons, but damage to nucleus, and presence of membrane-bound areas of disintegrating cytoplasm (arrows), is typical of KBV-affected cells. Bar = 2 µm.


A
B

5.3), with obvious damage being restricted to the glial and chiasmata cells. Figure 6.3 (b) shows bundles of axons, still with some glial investment, adjacent to areas of disintegrating cytoplasm (arrows), and otherwise surrounded by the featureless cytoplasm which typically results from KBV-induced destruction of glial cells. It appears that in mixed infections, replication of SBV in glial cells was effectively prevented by this extreme pathological response.

6.6 Discussion

The experiments reported in this chapter have shown that the outcome of mixed SBV and KBV infections depends on both the multiplicity of each infecting virus, and on the length of the delay before superinfection. Variation of these parameters showed that such infections could result in reduced multiplication of one or both viruses, or in the exclusion of SBV by KBV. The importance of these two factors has been reported from similar experiments in both whole animal and cell culture systems (for example, Kurstak et al., 1974 ; Alonso and Carrasco, 1982).

The decreased yields and rates of viral growth observed in this study indicate the existence of some interference mechanism, but because these experiments used whole animals, rather than cultured cell lines, further interpretation is difficult. It is not, for example, possible to determine whether this phenomenon resulted from a reduced rate of growth in individual cells, or from similar patterns of growth in a reduced number of cells.

Such interpretation as is possible rests largely on the histological evidence presented in this and earlier chapters. The demonstrated non-cytopathic effect of SBV in single infections (see Chapter 5) suggests that precocious tissue damage was an unlikely cause of yield reduction, and other evidence suggests that neither insufficient viral replication time, nor age-based differences in pupal susceptibility were likely to be responsible.

It is not, however, possible to rule out all potential causes of a non-specific nature. For example, the demonstrated ability of these unrelated viruses to grow in the same cell makes this experimental system similar to those previously reported by Cords and Holland (1964) and Alonso and Carrasco (1982). Both pairs of authors showed interference between co-replicating heterologous picornaviruses, the former attributing this result to competition for metabolites and replication sites, and the latter illustrating that changes in the extra-cellular osmotic conditions could markedly influence the relative amounts of virus produced. Such non-specific mechanisms might easily explain the status of infections induced by, respectively, very late or simultaneous KBV inoculation ; further, it is possible that variable combinations of such factors might produce the different types of cellular damage observed at sites of infection. It is presumed that damage to tissue of the nervous system was caused by the same mechanism suggested previously (see Chapter 5).

However, the possible involvement of a specific interfering mechanism between SBV and KBV should not be overlooked. The histological and physiological evidence presented in previous chapters suggests that SBV infection of bee pupae has a status similar to the symptomless,

persistent infections induced in mosquitoes and mosquito cells by various toga- and bunyaviruses ; both homologous and heterologous specific interference have been demonstrated in these latter systems (see, for example, Peleg, 1975 ; Davey et al., 1979).

The phenomena of phenotypic mixing and genomic masking during mixed infections of small RNA viruses have been well documented (Dodds and Hamilton, 1976), but for both practical and theoretical reasons were not investigated during the present study. On practical grounds, conclusive demonstration of the products of such viral interactions would be a formidable task. Bee viruses are apparently specific to A. mellifera (Longworth, 1978),so that there is no alternative host in which either virus could be differentially propagated. Further, small amounts of SBV RNA would be unable to establish infection of A. mellifera when inoculated simultaneously with KBV, and the appearance of KBV in SBV-inoculated pupae might easily result from undetectable levels of contamination of inocula (that is, less than 0.01% ; see Table 6.1). Because of the host specificity mentioned above, virus particles resulting from either type of interaction would, in any case, be of little field importance, since they would not lead to any extension of the natural host range.

Murphy (1975) suggested that viral interference might be partially responsible for modulation of arbovirus infection in mosquitoes, and the results presented here suggest that it might have a similar role in limiting the spread of honey bee viruses.

In this study, inoculum sizes and incubation times have both been consistent with known field values for such parameters, and at these

levels have been found capable of causing major repression of KBV replication. Further, it is known that a high proportion of apiaries in Australia are infected by SBV (Hornitzky, 1981 ; Dall, unpublished observations), and, as reported in Chapter 3 and by Bailey and Fernando (1972), that seemingly healthy pupae and adults can carry large amounts of that virus. It is then tempting to speculate that given sufficient establishment in a hive, the presence of SBV might reduce KBV replication to levels insufficient to precipitate epizootic infection.

CHAPTER 7 : SUSCEPTIBILITY OF TWO SMALL MAMMAL SPECIES TO SACBROOD AND KASHMIR VIRUSES

7.1 Introduction

The relationship between small RNA viruses of vertebrates and invertebrates has been a subject of discussion for many years (see Longworth, 1983), and several lines of evidence now indicate that, for some groups at least, it may be quite close.

While it is of interest that the invertebrate-infecting Nodamura virus can also infect both vertebrates (Scherer et al., 1968) and cultured vertebrate cells (Bailey et al., 1975), host range should probably not be considered a valid criterion when judging relationships of this sort. More important in this respect is work such as that of Tinsley et al., (1984) which demonstrated the existence of antigenic cross-reactivity between cricket paralysis virus (CrPV) and encephalomyocarditis virus (EMCV). In addition to supporting the notion of some relationship between the groups, such cross-reactivity may at least partially explain the presence of antibodies to cricket paralysis, Nodamura, Gonometa and Darna trima viruses which have been detected in sera from one or more of a wide range of vertebrates (Longworth et al., 1973 and Scotti et al., 1981). Bailey et al. (1979) suggested that cross-reactivity of this type might also account for antibodies to KBV which they detected in 'normal' rabbit and human sera.

To date, three invertebrate viruses [Gonometa, CrPV and Drosophila C virus (DCV)] have been accepted as members of the otherwise mammalian-specific Picornaviridae, and several other viruses, including SBV, are

considered potential members (Moore and Tinsley, 1982). In order to be assigned to that family, however, viruses must display the characteristic picornaviral replicative strategy (see Sangar, 1979) ; the failure of SBV to replicate in established insect cell lines (Bailey, 1976), coupled with the resistance of both Apis and other hymenopteran cells to in vitro culture, has so far prevented the demonstration of such a mechanism.

The similarities already noted between the intracellular structures induced during multiplication of SBV and TMEV (see Section 4.4.3) suggested that SBV, too, might replicate in mice, and that from this host it might be possible to adapt the virus to mouse cell lines. Unfortunately, as described below, this was found not to be so. Likewise, parallel attempts to infect mice with KBV also proved unsuccessful.

In Australia both feral mice and small marsupials are occasionally found seeking food and/or shelter in beehives (Fleay, 1949 ; Redpath, 1980) suggesting that they might be potential vectors of honey bee viruses. This was of particular interest in the case of marsupials, since Bailey (1981) has suggested that an element of the Australian fauna probably acts as a reservoir of KBV. Feeding trials with the stripe-faced dunnart, Sminthopsis macroura, suggested that this species, at least, was unlikely to play a role in the dissemination of either virus.

7.2 Materials and methods

Feeding trials with Sminthopsis macroura. Adult male S. macroura (Gould) were held individually in cages constructed of wood and flywire, measuring 30 x 30 x 60 cm. They were fed a varied diet of cooked minced lamb heart mixed with high protein baby cereal, chopped hard-boiled egg mixed with meat-based baby food and cereal, honey bee pupae and mealworm (Tenebrio molitor L.) larvae and pupae. The animals were kept under natural light conditions at a temperature of 20 to 31°C.

Honey bee pupae infected with either sacbrood or Kashmir virus were very readily eaten by the animals. Infected pupae were fed to two individuals on a single occasion, and two control animals were fed uninfected pupae. The animals exposed to SBV consumed a total of about 0.5 mg of virus, while those tested with KBV consumed about 2.5 mg.

Faeces were collected daily for three days prior to feeding with virus, and then for a further 14 days. Faeces were disrupted in 1 ml of KP buffer containing 0.05% (v/v) Tween 80, centrifuged at 8,000 *g* for 3 min, and a 1/20 dilution of supernatant was tested for virus using the ELISA method.

For serological testing, about 100 µl of blood was collected from the main caudal vein, and prepared as described (Section 2.6).

Infectivity of SBV and KBV for Mus musculus. The first vertebrate passage of each virus was in a litter of new-born outbred white mice (I.M.V.S. strain, Gilles Plains, South Australia); each animal was inoculated with either SBV or KBV as described (Section 2.4). Each mouse

received 5 µl of inoculum both intracranially and intraperitoneally, giving a total dose for each virus of about 1 µg/individual.

After various incubation times (see Table 7.1) inoculated mice were sacrificed, and the brain, heart, liver, spleen and small intestine was dissected from each cadaver. Half of each organ was tested for virus by ELISA, and the remaining material was pooled on the basis of KBV or SBV inoculation. The two group samples were each homogenised in 2 ml of ice-cold 2/3 IR / 1/3 KP buffer containing 500 units/ml of both penicillin and streptomycin, and 5 µl of homogenate was inoculated both intracranially and intraperitoneally into a second litter of new-born mice. Tissue from second passage mice was prepared and tested as described above.

7.3 Results

7.3.1 Sminthopsis macroura

After being fed infected pupae, three of the four animals produced faeces which were virus-positive by ELISA. Two animals (one KBV-fed, and the SBV-fed individual described below) each produced only one positive sample, being that collected within 24 hr of feeding, while the second KBV-fed animal was found to have excreted virus for up to 48 hr after feeding. It is assumed that failure to recover virus from faeces of the second SBV-fed animal was a consequence of the very low viral dose.

Daily observation of the animals detected only one instance of clinical illness, in an animal which had been fed SBV-infected pupae. This animal showed general lethargy, accompanied by conjunctivitis-like

symptoms such as discharge of gummy material, and puffy swelling around the eyes. These symptoms were first detected nine days after ingestion of the virus, and persisted for about 24 hr. A blood sample taken several days later (Day 13) was tested against SBV by double immunodiffusion, but serum antibodies were not detected ; on the basis of this finding, and the ELISA results reported above, it was concluded that the illness was not caused by exposure to the virus. All other animals remained healthy for the duration of the experiment.

7.3.2. *Mus musculus*

Table 7.1 shows the periods of incubation and numbers of mice sacrificed following each of the inoculations. Subsequent testing by ELISA showed that background readings differed markedly between, but not within, homogenates of the various types of organ ; in no instance, however, was virus replication apparent.

Daily observation of the animals detected no sign of chronic illness, and only one death was recorded during the experiment, occurring 14 days after inoculation with KBV. The cadaver of this animal was partly consumed by its mother, and the remainder was, unfortunately, destroyed. In the hope that a virulent KBV isolate might be infective *per os*, tissue samples were taken from the mother and tested serologically, but these, too, produced only negative results.

7.4 Discussion

The apparent failure of the viruses to replicate in mice, or in *S. macroura* following administration *per os*, agrees with previous claims that bee viruses are host specific (Longworth, 1978).

Table 7.1 : Details of attempted infection of new-born laboratory mice with SBV and KBV. See text for experimental methods.

PASSAGE	PASSAGE DETAILS			SBV TESTS		KBV TESTS	
	Inoculation date	Sampling date	Growth time (days)	Number tested	Notes	Number tested	Notes
1	27.11.84	17.12.84	20	4	Siblings; Litter 'A'	2	Siblings; Litter 'B'
		4. 1.85	38	2	Siblings; Litter 'A'	3	Mother and siblings; Litter 'B'
2	17.12.84	4. 1.85	18	4	Siblings; Litter 'C'	4	Siblings; Litter 'D'

It is possible that the inoculated viruses were not given sufficient opportunity to adapt to Mus musculus ; this study involved only two viral passages, while other small RNA viruses have frequently required large numbers of passages before their adaptation to alternate hosts.

Whole animals were initially chosen for this work because it was thought that exposure of virus to a diversity of cell types might increase the chance of establishment of infection. It now seems that future work might be more conveniently done by using a variety of cell lines, given that a large number of blind passages are likely to be required. Alternatively, methods such as 'single-round' infection of cell cultures with naked viral RNA, or translation of such RNA in artificial systems are probably more likely to lead to understanding of the replicative strategies of these viruses.

The speed with which the marsupials eliminated each virus from their alimentary system is in keeping with their documented rapid digestion rate (Hume, 1982). This factor probably diminishes the chance of establishment of accidental infection, and suggests that these animals are unlikely to be responsible for wide dissemination of either virus, even by a simple phoretic mechanism.

CHAPTER 8 : GENERAL DISCUSSION

This study has examined a number of aspects of the infective and multiplicative processes of sacbrood and Kashmir bee virus growth in pupae of Apis mellifera L. The results of the various investigations have been both discussed and compared with relevant literature reports at other places in the thesis ; the purpose of this chapter is to provide a brief overview of these results, and to outline some potentially profitable directions for future work.

In retrospect, the choice of these two viruses for detailed comparison was a fortuitous one. At every turn, SBV and KBV have shown wide divergence in their multiplicative capabilities, and in their response to similar experimental conditions, and have thus provided graphic illustration of the manner in which the consequences of viral infection may vary, even in a single host species.

The complex range of responses observed in cells infected by either virus is in sharp contrast to the few recognised, or recognisable, external signs of infection. These visible symptoms are generally of such a gross or dramatic nature that their recognition provides no real indication of the underlying causative mechanism.

The results presented in this study show that SBV infection had no detectable pathological consequences for the pupal host. In contrast, overt infection with KBV seemed invariably to lead to rapid perturbation of host physiology, major direct and indirect cytopathological damage, and to culminate in death of the host.

When results from laboratory and field studies are taken in conjunction, it seems that these differences reflect variation in the mechanisms of persistence adopted by each virus. While inapparent infections of SBV have been reported both here and elsewhere, results presented earlier in this thesis suggest that this does not represent a specialised strategy of persistence. Rather, this inapparent status seems to reflect the effectively symptomless nature of such infections. All evidence suggests, however, that inapparent and overt infections of KBV have very different status, though it is not known whether the former represents a very closely-regulated state of the latter, or is due to an entirely different mechanism, perhaps similar to a state of true viral latency, sensu Mims (1982).

Comparison of material published by Bailey et al. (1964, 1965) with findings from this present study suggests that KBV and the unrelated acute bee paralysis virus (APV) might occupy very similar pathogen niches in the Australasian and Palaearctic/Nearctic regions, respectively. KBV has never been recorded from bees in Europe or the Americas, and while APV is present in Australia, it is apparently very rare. In the appropriate region each virus persists in an 'inapparent' state, causing occasional field losses during localised epizootics. In laboratory studies, however, each has now been shown to be a highly infective and virulent pathogen, with near identical levels of infectious dose and yield. Further, the gross symptomology seen in overt infections of these viruses is similar in both appearance and time of onset, although, as mentioned above, the diagnostic value of this character is uncertain. Finally, of all viruses isolated from Apis mellifera, KBV and APV are the only two in which a number of serologically distinguishable strains have been demonstrated.

Given these similarities, study of the interactions between KBV, APV and SBV might significantly advance the understanding of factors which influence viral disease in honey bees. Demonstration of a semi-permissive interaction between APV and SBV, similar to the type shown here for KBV and SBV, would strengthen the hypothesis that interactions between bee viruses play an important role in determining their field distribution. Additionally, it could prove interesting to compare results from detailed microscopical investigations of APV replication with those of KBV, as presented here.

In summary, it seems that the study of viral diseases of honey bees is now at a critical point. The massive task of identification of important viral pathogens has, in the main, been completed, although there is little doubt that other viruses remain to be described. The known array of 14 different viruses, with, in some cases, a number of serological strains, would appear to qualify this as an excellent system for comparative investigation of invertebrate virus replication, viral interaction and virus/host relationships. This potential is, however, frustrated by the lack of an Apis-derived cultured cell line ; to date, all work has used various developmental stages of live bees. The problems generally associated with study of virus growth in whole animals are exacerbated in this situation by the social habits of the insect, and by its 'free-range' requirement, both of which prevent the use of 'specific pathogen free' hosts. Given appropriate controls these problems do not, I believe, compromise the value of work dealing with histological and 'higher order' studies of viral growth, but they entirely preclude study of topics which require synchronous infection of host cells.

In my opinion, culture of cells capable of supporting bee virus replication is of paramount importance if this field is to continue to develop. Results from such a cell culture system, when taken in conjunction with those from other laboratory and field studies, would offer an unrivalled opportunity to understand the complexities of the relationship between an important insect host and its viral pathogens.

APPENDIX A : RELATIONSHIP BETWEEN VIRUS PARTICLE NUMBERS AND VIRUS WEIGHTS

Molecular weights of viral coat proteins were determined electrophoretically using the method of Laemmli (1970). The means and standard errors quoted below are based on five determinations in 10, 12 or 15% acrylamide gels calibrated with Pharmacia molecular weight standards. For SBV, computed protein weights (x 10^3) were 38.1 ± 0.6, 34.8 ± 0.8 and 31.4 ± 0.4 daltons, while for KBV they were 39.6 ± 0.6, 37.7 ± 0.5 and 25.3 ± 0.5. In no instance was a smaller protein (i.e. VP4) detected. The values for KBV agree closely with other estimates of proteins from the Queensland 2 strain (Bailey et al., 1979), but the values for SBV are markedly different from those previously published (Bailey, 1976). The reason for this discrepancy is not clear.

Based on the above values, the summed coat protein weight of each virus is assumed to be about 100,000 daltons. Allowing 60 copies of each protein per capsid (see Putnak and Phillips, 1981), and an RNA of 2.8 x 10^6 daltons (Bailey, 1981) the total molecular weight of each particle is in the vicinity of 8.8 x 10^6 daltons. One mole of particles (6.023 x 10^{23}) thus weighs 8.8 x 10^6 gm, so that an individual particle weighs 1.46 x 10^{-17} gm. On this basis 1 ng of virus ≡ 6.85 x 10^7 particles.

The particle numbers used in Section 4.2 have been derived from this value, but it should be remembered that this is an approximation, itself based on a number of assumptions, and thus must be treated with caution.

APPENDIX B : PUBLISHED PAPER

DALL, D.J. (1985). Inapparent infection of honey bee pupae by Kashmir and sacbrood bee viruses in Australia.

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