

INFECTIVITY OF SOME OCCLUDED INSECT VIRUSES

by

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I dedicate this thesis to my parents, who
have provided constant encouragement and
support for my studies.

ABSTRACT

The inclusion body (I.B.) structure and biological properties of six occluded insect viruses were studied comparatively. The viruses chosen were five baculoviruses and a cytoplasmic polyhedrosis virus (CPV) which are pathogenic to the larval stages of several lepidopterous pests of temperate agriculture. The baculoviruses included two granulosis viruses (GVs), two nuclear polyhedrosis viruses with multiply enveloped virus particles (mNPVs) and one singly enveloped NPV.

The viruses were partly characterized by electrophoretic analysis of RNA genome segments (CPV) or restriction enzyme digests of viral DNA (baculoviruses). Virus particle and I.B. polypeptides were also examined. Four of the viruses had not been previously analysed by these techniques, which are essential for identification of inocula and progeny virus in cross-transmission tests. Comparison of three European isolates of one of the viruses (Agrotis segetum NPV) revealed that two isolates were identical and the third completely distinct. Serological tests confirmed these relationships and, when applied to the other viruses, supported the accepted subgrouping of NPs and GVs. Packaging arrangements of nucleocapsids and virus particles within I.B.s showed wide variations. A. segetum mNPV (English isolate) had the largest number (670) of nucleocapsids per I.B. of the baculoviruses tested. Despite the large variations in this characteristic, the oral LD₅₀ of most viruses to neonate larvae of the homologous host was about 10 I.B.s. The main exception was A. segetum GV with an oral LD₅₀ of 10^4 capsules to A. segetum neonates.

The host range of each virus was tested in seven host species and in most cases the infectivity was examined. The CFV was the only virus infecting both larval families tested (Noctuidae and Pieridae). Mamestra brassicae NPV had the widest host range amongst the baculoviruses. In general heterologous infections had a higher LD50 value.

Dissolution of I.B.s was compared in vitro and studied in vivo using radio-labelled virus. The rate and extent of virus particle release from I.B.s did not appear to limit infectivity.

Electron microscopical examination of larval gut sections after virus feeding suggested that virus particle uptake at the gut epithelium may be a limiting factor in infection.

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TABLE OF CONTENTS

	<u>PAGE</u>
ABSTRACT	3
ACKNOWLEDGEMENTS	5
TABLE OF CONTENTS	6
LIST OF FIGURES	13
LIST OF TABLES	17
INTRODUCTION	18
CHAPTER 1 LITERATURE REVIEW	22
(1) THE OCCURRENCE OF OCCLUDED VIRUSES	23
(2) PHYSICAL, CHEMICAL AND ANTIGENIC PROPERTIES	25
a) BACULOVIRUSES	25
(i) STRUCTURE	25
(ii) RELATIONSHIPS BETWEEN	27
AND WITHIN SUBGROUPS	
b) CYTOPLASMIC POLYHEDROIC VIRUSES	32
c) ENTOMOPOXVIRUSES	33
(3) INCLUSION BODY DESIGN	33
(4) PATHOLOGY OF INFECTION BY OCCLUDED VIRUSES	38
a) INCLUSION BODY DISSOLUTION	38
b) VIRUS SURVIVAL IN THE GUT LUMEN	40
c) THE PERITROPHIC MEMBRANE AS A BARRIER	43
TO INFECTION	

	<u>PAGE</u>
CHAPTER 1	
(4) d) ATTACHMENT AND PENETRATION	44
e) PATHWAYS OF REPLICATION	45
(i) BACULOVIRUSES	45
(ii) CYTOPLASMIC	47
POLYHEDROSIS VIRUSES	
f) LATENCY	47
(5) HOST RANGE	50
(6) ORAL INFECTIVITY	53
a) DOSE STUDIES	53
b) TIME COURSE STUDIES	60
c) RESISTANCE TO VIRAL INFECTION	63
(7) VIRUS ECOLOGY	65
a) NATURAL INCIDENCE	65
b) TRANSMISSION AND SURVIVAL	67
CHAPTER 2	
MATERIALS AND METHODS	71
(1) DECONTAMINATION PROCEDURES	73
(2) THE INSECTS	74
a) SPECIES STUDIED AND ORIGIN OF STOCKS	74
b) MAINTENANCE OF INSECT STOCKS	74
c) DIET COMPOSITION	77
(3) THE VIRUSES	78
(4) PRODUCTION AND PURIFICATION OF VIRUSES	78
a) OCCLUDED VIRUS PREPARATIONS	78
b) SEPARATION AND PURIFICATION OF BACULOVIRUS	82
VIRUS PARTICLES, GRANULIN AND POLYHEDRIN	

	<u>PAGE</u>
CHAPTER 2 (5) LIGHT MICROSCOPY	83
a) EQUIPMENT	83
b) COUNTING INCLUSION BODIES	83
c) MEASURING POLYHEDRAL DIAMETERS	84
d) EXAMINATION OF LARVAL SMEARS	84
(6) ELECTRON MICROSCOPY	85
a) SPECIMEN PREPARATION FOR THIN SECTIONING	85
b) SECTIONING AND EXAMINATION OF THIN SECTIONS	86
c) NEGATIVE STAINED PREPARATIONS	86
d) SCANNING ELECTRON MICROSCOPY	86
(7) BIOCHEMICAL ANALYSES	87
a) SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF POLYPEPTIDES	87
b) NUCLEIC ACID PURIFICATION	88
c) RESTRICTION ENDONUCLEASE DIGESTION OF BACULOVIRUS DNA	89
d) AGAROSE GEL ELECTROPHORESIS OF NUCLEIC ACIDS	90
e) POLYACRYLAMIDE GEL ELECTROPHORESIS OF NUCLEIC ACIDS	91
(8) SEROLOGICAL ANALYSES	92
a) PREPARATION OF ANTISERA	92
b) IMMUNODIFFUSION	93
c) PREPARATION OF γ -GLUCULIN	93
d) ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)	93

	<u>PAGE</u>
CHAPTER 2 (9) INFECTIVITY ASSAYS	94
a) DOSING METHOD	94
b) ESTIMATION OF DOSE VARIABILITY ON LEAF DISCS USING ^{32}P -LABELLED VIRUS	96
(10) PRODUCTION AND COUNTING OF ^{32}P - AND ^3H -LABELLED VIRUS	97
CHAPTER 3 BIOCHEMICAL AND SEROLOGICAL ANALYSES OF THE VIRUS ISOLATES	100
(1) ELECTROPHORETIC PROFILES OF RESTRICTION ENDONUCLEASE DIGESTS OF BACULOVIRUS DNA'S	101
a) THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV	101
b) <u>M. BRASSICAE</u> NPV	104
c) <u>A. SEGETUM</u> GV	106
d) <u>P. GAMMA</u> sNPV and mNPV	107
(2) ELECTROPHORETIC PROFILE OF <u>P. GAMMA</u> CPV GENOME SEGMENTS	111
(3) SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF STRUCTURAL POLYPEPTIDES FROM THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV AND <u>M. BRASSICAE</u> NPV	113
a) POLYHEDRINS	113
b) VIRUS PARTICLE STRUCTURAL POLYPEPTIDES	118
(4) SEROLOGICAL ANALYSIS OF THE BACULOVIRUSES	120
a) SPECIFICITY OF ANTISERA	120
b) ANTIGENIC RELATIONSHIPS BETWEEN THE BACULOVIRUSES, DETERMINED BY INDIRECT ELISA	124
(5) DISCUSSION	129

	<u>PAGE</u>
CHAPTER 4 INCLUSION BODY STRUCTURE	132
(1) INCLUSION BODY MORPHOLOGY	133
(2) INTERNAL STRUCTURE OF INCLUSION BODIES	137
(3) CALCULATION OF INCLUSION BODY VOLUME	150
(4) CALCULATION OF NUMBERS OF NUCLEOCAPSIDS AND VIRUS PARTICLES WITHIN INCLUSION BODIES	155
a) NUMBER OF NUCLEOCAPSIDS PER VIRUS PARTICLE	156
b) NUMBER OF NUCLEOCAPSIDS AND VIRUS PARTICLES PER I.B.	158
(5) DISCUSSION	161
CHAPTER 5 HOST RANGE STUDIES	164
(1) INFECTION TESTS WITH NEONATE LARVAE	165
(2) IDENTIFICATION OF PROGENY VIRUS IN CROSS-INFECTION TESTS	166
a) BACULOVIRUSES	166
b) CYTOPLASMIC POLYHEDROSIS VIRUSES	173
(3) SUSCEPTIBILITY OF <u>M. BRASSICAE</u> TO THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV	176
(4) DISCUSSION	177
CHAPTER 6 STUDIES OF VIRUS INFECTIVITY	179
(1) LD50 ASSAYS	180
(2) LT50 CALCULATIONS	186
(3) VIRUS PRODUCTION ASSAY	188
(4) DISCUSSION	189

	<u>PAGE</u>
CHAPTER 7 INCLUSION BODY DISSOLUTION	194
(1) <u>IN VITRO</u> DISSOLUTION	195
(2) DISSOLUTION OF RADIOLABELLED I.B.'S <u>IN VIVO</u>	196
(3) ELECTRON MICROSCOPIC EXAMINATION OF DISSOLUTION <u>IN VIVO</u>	202
(4) DISCUSSION	209
 CHAPTER 8 GENERAL DISCUSSION	 211
(1) INFECTIVITY OF VIRUS PARTICLES AND NUCLEOCAPSIDS	212
(2) I.B. STRUCTURE AND INFECTIVITY	215
(3) I.B. POPULATION ECOLOGY	218
(4) PHYSIOLOGICAL ASPECTS OF INFECTION	220
(5) THE SIGNIFICANCE OF INFECTION PARAMETERS FOR MICROBIAL CONTROL OF INSECT PESTS	221
 SUMMARY	 223
 APPENDIX 1 A COMPARISON OF THE ELECTROPHORETIC MOBILITIES OF VIRAL ds RNA SEGMENTS AND ds DNA RESTRICTION ENDONUCLEASE FRAGMENTS, ON THREE GEL SYSTEMS	 226
(1) CPV AND <u>P. CHRYSOGENUM</u> VIRUS ds RNA SEGMENTS	227
(2) BACULOVIRUS AND λ PHAGE DNA FRAGMENTS	233
(3) EFFECT OF CHANGES IN GEL BUFFER SYSTEMS	234
(4) DISCUSSION	234

	<u>PAGE</u>
APPENDIX 2 STATISTICAL APPENDIX TO CHAPTER 4	236
BIBLIOGRAPHY	240

LIST OF FIGURES

	<u>PAGE</u>
2.1 Histogram of bioassay dose distribution.	98
3.1 Electrophoretic profiles of DNA, digested with EcoRI, from three European isolates of <u>A. segetum</u> NPV.	102
3.2 Electrophoretic profiles of DNA, digested with Hind III, from three European isolates of <u>A. segetum</u> NPV.	103
3.3 Electrophoretic profiles of DNA, digested with EcoRI, from <u>M. brassicae</u> NPV and <u>A. segetum</u> GV.	105
3.4 Electrophoretic profiles of DNA, digested with EcoRI, from <u>P. gamma</u> mNPV and <u>A. californica</u> NPV.	109
3.5 Electrophoretic profile of DNA, digested with EcoRI, from <u>P. gamma</u> sNPV.	110
3.6 Electrophoretic profiles of <u>P. gamma</u> CPV and <u>B. mori</u> CPV genome segments on 3% acrylamide gel.	112
3.7 Polyacrylamide gel electrophoresis of polyhedrins.	115
3.8 Co-electrophoresis of <u>M. brassicae</u> NPV and <u>A. segetum</u> (E) NPV polyhedrins.	116
3.9 Electrophoresis of polyhedrins, showing varying degrees of alkaline protease breakdown.	117
3.10 Electrophoresis of virus particle structural polypeptides from <u>M. brassicae</u> NPV and three European isolates of <u>A. segetum</u> NPV.	119

	<u>PAGE</u>
3.11 Immunodiffusion tests of antisera specificity.	122
3.12 Reaction of <u>M. brassicae</u> NFV antisera with dissolved NFV I.B.'s tested by double antibody sandwich ELISA.	123
3.13 Reaction of NFV antisera with dissolved NFV and GV I.B.'s, tested by indirect ELISA.	126
3.14 Reaction of <u>A. segetum</u> (E) NFV antisera with dissolved NFV and GV I.B.'s, tested by indirect ELISA.	127
3.15 Reaction of NFV and GV antisera with dissolved NFV and GV I.B.'s, tested by indirect ELISA.	128
4.1 Scanning electron microscopy of viral inclusion bodies.	134
4.2 Phase contrast microscopy of <u>A. segetum</u> (E) NFV I.B.s	136
4.3 Thin sections of <u>A. segetum</u> GV I.B.'s.	139
4.4 Thin sections of <u>A. segetum</u> GV, aberrant I.B.'s.	140
4.5 Thin sections of <u>P. brassicae</u> GV I.B.'s.	141
4.6 Thin sections of <u>P. gamma</u> sNFV I.B.'s.	142
4.7 Thin sections of <u>P. gamma</u> mNFV I.B.'s.	143
4.8 Thin sections of <u>M. brassicae</u> NFV I.B.'s and <u>A. segetum</u> (F) NFV I.B.'s.	144
4.9 Thin sections of <u>A. segetum</u> (E) NFV I.B.'s.	145
4.10 Thin sections of <u>P. gamma</u> CFV I.B.'s.	146

4.11	Negative stained preparations from alkaline dissolved NPV I.B.'s.	147
4.12	Negative stained preparations of <u>P. gamma</u> sNPV, partly dissolved I.B.'s.	148
4.13	Isopycnic sucrose gradient centrifugation of dissolved <u>M. brassicae</u> NPV and <u>A. segetum</u> (E) NPV I.B.'s.	149
4.14	Relationship between I.B. volume and protein content for six NPV preparations.	154
5.1	Electrophoretic profile of <u>M. brassicae</u> NPV virus particle polypeptides, grown in <u>A. segetum</u> , <u>M. brassicae</u> and <u>A. exclamationis</u> .	169
5.2	Electrophoresis of baculovirus inocula and progeny DNA, EcoRI digests on 1% agarose gel.	170
5.3	Phase contrast microscopy of smeared larvae: NPV infection of <u>M. pronuba</u> and CPV infection of <u>P. gamma</u> .	172
5.4	Electrophoresis of CPV inocula and progeny RNA segments on 3% acrylamide gel.	174
5.5	Electrophoresis of CPV inocula and progeny RNA segments on 10% acrylamide gel.	175
7.1	Alkaline dissolution of baculovirus I.B.'s <u>in vitro</u> .	197
7.2	Dissolution of ³² P-labelled <u>A. segetum</u> (E) NPV I.B.'s <u>in vivo</u> .	199

	<u>PAGE</u>
7.3 Dissolution of ³ H-labelled I.B.'s <u>in vivo</u> .	200
7.4 I.B. dissolution <u>in vivo</u> . Thin sections of larvae.	203
7.5 I.B. dissolution <u>in vivo</u> . Thin sections of larvae.	204
7.6 Midgut sections of <u>M. brassicae</u> neonates fed <u>M. brassicae</u> NPV, showing undissolved I.B.'s.	205
7.7 Midgut of <u>M. brassicae</u> neonates fed <u>M. brassicae</u> NPV.	206
7.8 Midgut of <u>M. brassicae</u> neonates fed <u>M. brassicae</u> NPV.	207
7.9 Haemocoel of <u>P. rapae</u> neonate infected with <u>P. brassicae</u> GV.	208
A.1 Electrophoresis of nucleic acids on 1% agarose.	229
A.2 Electrophoresis of nucleic acids on 1% agarose.	230
A.3 Electrophoresis of nucleic acids on 3% polyacrylamide.	231
A.4 Electrophoresis of nucleic acids on 10% discontinuous polyacrylamide.	232

LIST OF TABLES

	<u>PAGE</u>
1. The lepidopterous species studied.	75
2. Origin of virus isolates studied.	79
3. Polyhedral size measurements.	152
4. Distribution of no. nucleocapsids per virus particle in <u>A. segetum</u> (E) NPV, <u>A. segetum</u> (F) NPV and <u>M. brassicae</u> NPV.	157
5. No. virus particles and nucleocapsids per inclusion body.	160
6. Virus host range.	162
7. Complete results of LD50 assays.	182
8. Comparative LD50's.	184
9. LT50 results.	187
10. LD50's for virus infections, in neonate larvae, converted to equivalent numbers of virus particles (CPV) and nucleocapsids (baculoviruses).	213

INTRODUCTION

Three virus families, the Baculoviridae, Reoviridae and Poxviridae include some members in which the infection cycle terminates in the production of occluded infectious virus. Here the virus particles are enclosed within a protein crystal of viral origin (the inclusion body or I.B.). These crystals appear to be the main vehicle for virus transmission to new host individuals. Other viruses in these three families are not occluded in this sense, notably the vertebrate reoviruses and poxviruses.

The hosts of occluded viruses are almost exclusively larvae of the insect families Diptera, Hymenoptera, Coleoptera and, most frequently, Lepidoptera.

Considering the divergent characters of the viruses involved, it is probable that the I.B. is a feature of convergent evolution. However, its function is poorly understood. It is commonly believed that the I.B. has a role in protecting virus particles while outside the host. Indirect evidence for this hypothesis is provided by studies on the persistence of non-occluded and occluded viruses (Chapter 1, Section 7(b)). The fact that occluded viruses mainly infect larval stages of holometabolous Insecta also adds circumstantial support to the protection hypothesis, for in these species the virus must survive for periods when there are no available hosts (Chapter 1, Section 1). There is some evidence that the I.B. may reduce degradation of virus particles during the process of oral infection but this is inconclusive (Chapter 1, Section 4(b)).

If either or both of these factors are important they are still only explanations of the presence of the I.B., not the structural variations which it exhibits. Most occluded viruses enclose more than one infective unit in each I.B., which therefore acts as a 'package' of inoculum. Perhaps this is the main role of the I.B., and forms with only one virus particle in each I.B. (notably granulosis viruses and some cytoplasmic polyhedrosis viruses) might be special cases of the general condition. Very little work has been done to compare the structural forms of occluded viruses or explain the variation found. While it is known that the number of occluded infective units per I.B. varies markedly between different viruses (particularly among lepidopteran baculoviruses), there have been no studies on the relationship between this characteristic and the virus' biological properties. In particular it is not known how infectivity relates to the number of virus particles occluded within the I.B.

It is the purpose of this study to examine this relationship by analysing inclusion body structures of a range of occluded viruses, and their infectivity to different hosts. Five baculoviruses were chosen which exhibit the three major forms of virus packaging: two granulosis viruses (GV), one nuclear polyhedrosis virus in which all the nucleocapsids are singly enveloped (SNPV) and two nuclear polyhedrosis viruses where one or more nucleocapsids are found in each virus particle (MNPV). A cytoplasmic polyhedrosis virus (CPV) was examined in comparison. A method for measuring the numbers of nucleocapsids in each type of inclusion body was developed and the structures of the viral I.B.'s were compared. This comparison was then related to the viruses' biological properties of infectivity and host range.

The viruses used were originally isolated from important agricultural pests. In this study an examination was made of their infectivity to seven pest species including the original hosts. By measuring infectivity at a standard larval stage (neonate), the results using different species are directly comparable. Two criteria were used in choosing species for this analysis. First, they exhibit a variety of taxonomic relationships, which is useful for host-range studies. Second, they have overlapping geographical ranges and are often found on the same food plants (Table 1). The viruses are therefore in a position to compete for the same hosts. The host range study shows how each virus is adapted to a specific niche within this ecosystem. Such an analysis is useful in the assessment of viral agents for pest control, particularly where one pest is susceptible to several viruses. The host spectrum of a virus must be thoroughly studied to determine its possible value in controlling several pests and for its effect on non-target species.

The mechanisms for differences in virus virulence in homologous and heterologous hosts are unknown. It is clear that I.B.'s of different viruses require more or less extreme conditions for dissolution, possibly as an adaptation to conditions in the host gut. Whether the rate or extent of dissolution in alternative hosts limits infectivity has not been examined. In this thesis a quantitative method was devised to examine this question. If the I.B. functions as a bag of infectious particles which are released equally in different hosts, the virulence differences may reside in the survival of virus in the gut lumen, or in the rate of uptake at the gut epithelium. These possibilities were examined together with the data on absolute infectivity to see if a clear pattern emerged. Clearly, virulence may be influenced by other molecular characteristics, but studies of these did not form part of the present investigation.

For the results of cross-transmission studies to be valid it is essential to identify the inoculum and progeny viruses. Published studies are often meaningless because they have not fulfilled this criterion. In this project identification was achieved by electrophoretic analysis of RNA genome segments (CPV) or DNA fragments produced by restriction endonuclease digestion of the viral genome (baculoviruses). Relationships between viruses were examined using serology and polypeptide electrophoresis. Recently, workers have shown that virus isolated from one species in different geographical areas may exhibit genomic and biological variation (Chapter 1, Sections 2 and 6). In this study one of the major pest species examined was Agrotis segetum, the turnip moth. Three european isolates of NPV isolated from this species were examined to determine their relatedness and infectivity to A. segetum.

CHAPTER 1 LITERATURE REVIEW

<u>CONTENTS</u>	<u>PAGE</u>
(1) THE OCCURRENCE OF OCCLUDED VIRUSES	23
(2) PHYSICAL, CHEMICAL AND ANTIGENIC PROPERTIES	25
a) BACULOVIRUSES (i) STRUCTURE	25
(ii) RELATIONSHIPS BETWEEN AND WITHIN SUBGROUPS	27
b) CYTOPLASMIC POLYHEDROSIS VIRUSES	32
c) ENTOMOPOXVIRUSES	33
(3) INCLUSION BODY DESIGN	33
(4) PATHOLOGY OF INFECTION BY OCCLUDED VIRUSES	38
a) INCLUSION BODY DISSOLUTION	38
b) VIRUS SURVIVAL IN THE GUT LUMEN	40
c) THE PERITROPHIC MEMBRANE AS A BARRIER TO INFECTION	43
d) ATTACHMENT AND PENETRATION	44
e) PATHWAYS OF REPLICATION	45
(i) BACULOVIRUSES	45
(ii) CYTOPLASMIC POLYHEDROSIS VIRUSES	47
f) LATENCY	47
(5) HOST RANGE	50
(6) ORAL INFECTIVITY	53
a) DOSE STUDIES	53
b) TIME COURSE STUDIES	60
c) RESISTANCE TO VIRAL INFECTION	63
(7) VIRUS ECOLOGY	65
a) NATURAL INCIDENCE	65
b) TRANSMISSION AND SURVIVAL	67

CHAPTER 1

LITERATURE REVIEW

(1) The Occurrence of Occluded Viruses

Harrap and Payne (1979) list eight groups of viruses which contain members pathogenic to insects and other arthropods.

FAMILY OR GROUP	OTHER NAMES	ABBREVIATION	NUCLEIC ACID
1) BACULOVIRIDAE*			
SUBGROUP A	NUCLEAR POLYHEDROSIS	NPV	ds DNA
SUBGROUP B	GRANULOSIS	GV	ds DNA
SUBGROUP C	<u>ORYCTES</u> VIRUS GROUP		ds DNA
2) REOVIRIDAE	CYTOPLASMIC POLYHEDROSIS	CPV	ds RNA
3) POXVIRIDAE	ENTOMOPOXVIRUSES	EPV	ds DNA
4) IRIDOVIRIDAE	IRIDOVIRUSES		ds DNA
5) PARVOVIRIDAE	DENSOVIRUSES		ss DNA
6) RHABDOVIRIDAE			ss RNA
7) SMALL RNA VIRUSES			ss RNA
8) 'UNCLASSIFIED' E.G. <u>DROSOPHILA</u> X			ds RNA

Occluded viruses occur only in groups one to three. Within these three groups non-occluded viruses may also occur, notably the Oryctes virus (Huger, 1966) and some putative baculoviruses of crustaceans (Pappalardo & Bonami, 1979; Sano et al., 1981). The relative abundance of the occluded viruses can be judged from the report by Martignoni and Iwai (1981). This is summarised below as modified by the comments of Rohrmann et al. (1981) and Payne (1981b).

* A variety of parasitoid wasps have viruses associated with their ovaries (Stoltz and Vinson, 1979). These are probably not baculoviruses (Krell and Stoltz, 1981) and are not included here.

Number of Reports of Infections by Occluded Viruses

? indicates the report is dubious

<u>HOST CLASS/ORDER</u>	<u>VIRUS</u>			
	BACULOVIRUS		CPV	EPV
	NPV*	GV		
CRUSTACEA:DECAPODA	1	-	1	-
INSECTA:ORTHOPTERA	5?	-	-	-
NEUROPTERA	2?	2?	-	-
COLEOPTERA	2?	-	2?	17
DIPTERA	21	-	17	5
LEPIDOPTERA	337	103	163	18
TRICHOPTERA	1	-	-	-
HYMENOPTERA	25	1?	7	-

The figures in this table are to some extent a reflection of the amount of research effort which has been put into each host order as a result of its pest status. Many workers have failed to positively identify the virus involved and so some reports involve the same virus in several different hosts.

The occurrence of occluded viruses is limited to arthropod hosts of which the majority are holometabolous Insecta. Of the insect orders in the table above only the Orthoptera is hemimetabolous. In other words most of these viruses infect the larval stages of insects where the adult stage is considerably different in structure and habits. Such a host is only available to the virus for limited periods of time between which it must survive outside the host system. In this context it is interesting

* NPV's may be singly or multiply enveloped (sNPV or mNPV) as described in Section 2(a)(i) and 3. mNPV's are only found in lepidopterous hosts.

to note that the non-occluded baculovirus of Oryctes rhinoceros infects adult as well as larval beetles and so has a continuous supply of hosts (Section 7(b)). The only known baculovirus found outside the insects is the NPV of the shrimp Penaeus duorarum (Couch, 1974). There is insufficient information on the ecology of this virus to decide if it must survive outside the host in the same way that insect occluded viruses must.

Nearly all entomopathogenic viruses from the families Baculoviridae, Reoviridae and Poxviridae are occluded, suggesting that this is a successful adaptation to insects as hosts. However it is possible that more non-occluded viruses from these families remain to be discovered, particularly if they produce sub-lethal infections.

The widest range of structural forms is found among baculoviruses of Lepidoptera. There are no definite reports of GV or mNPV outside this order.

(2) Physical, Chemical and Antigenic Properties

Most attention will be paid to the baculoviruses and to a lesser extent the CPVs, as these are the virus groups examined in the present study. The literature on entomopoxviruses (EPV's) is very limited and will be mentioned briefly where it is of comparative interest.

a) Baculoviruses

(i) Structure. The structure of these viruses has been comprehensively reviewed by Harrap and Payne (1979) and Summers (1977). The nucleocapsid is rod shaped, with dimensions of 30-50 nm by 220-320 nm. It may be somewhat compressed within the viral envelope (Brown et al., 1977). The virus particle dimensions vary widely. One or more nucleocapsids

may be enveloped together, multiply enveloped forms being found mainly in the mNPV's but also in some GV's (Section 3). In addition dimensions of the virus particles differ when occluded as opposed to alkali-liberated virus is measured (Harrap, 1972(b)). Figures ranging from 40-140 nm by 250-400 nm are commonly found. The enclosed ds DNA is in the form of a covalently closed circle of molecular weight $80 - 100 \times 10^6$. A recent study by Kelly and Brown (1980) suggests a somewhat higher molecular weight of 115×10^6 for the DNA of Mamestra brassicae NPV.

The virion contains 11-25 polypeptides when analysed by SDS polyacrylamide gel electrophoresis and the nucleocapsid 4-8, after liberation from the lipoprotein envelope by the action of non-ionic detergent (Harrap and Payne, 1979). Results of gel electrophoresis vary due to the action of one or more alkaline proteases which are present in virus produced in larvae. These enzymes are not found in tissue culture-derived virus (Zummer and Faulkner, 1979) and so are presumably not of viral origin. The infectivity to larvae of tissue culture-derived and larval-derived NPV has been shown to be the same, suggesting that the protease is of no importance in infection (Faulkner and Henderson, 1972; Vail et al., 1973; Ignoffo et al., 1973(a)). The release of virus particles from larval-derived I.B.'s by the action of dilute alkali results in the degradation of some polypeptides due to the proteases. This is one explanation of the different profiles found on gel electrophoresis of viral polypeptides where I.B.'s have been dissolved under different conditions. Examples of this are the electrophoretic profiles of M. brassicae NPV virus particle polypeptides found by Kelly and Brown (1980) and Maskos and Miltenburger (1981).

Baculoviruses of subgroups A and B produce an occluded form of virus towards the end of the infection cycle (Section 4e). The I.B. acts as the unit for the transmission of virus by oral infection of further host individuals. It consists of a paracrystalline protein with polypeptide subunit molecular weight $25 - 31.4 \times 10^3$, in which the virus particles are embedded. This protein, known as granulin or polyhedrin in the case of GV's and NPV's respectively, has been shown to be virus specified in Autographa californica NPV infection (Van der Beek et al., 1980). The evidence for a surface membrane on the I.B. is summarised by Summers (1977). This appears to consist mainly of carbohydrates (Minion et al., 1979).

(ii) Relationships between and within subgroups. The division of Baculoviridae into three subgroups is based almost entirely on the presence or absence and structure of the I.B. as discussed in Section 3. Detailed examination of biochemical and serological data shows little evidence that viruses within subgroups are more closely related than between subgroups and this evidence is considered here.

Upholt (1977) notes that differences between polypeptides can be analysed by three techniques depending on the scale of difference

- (1) electrophoretic comparison for closely related polypeptides,
- (2) antigenic similarity for moderately close relationships,
- (3) amino acid sequence for distant relationships.

Even where very similar phenotypes are produced there may be significant genetic variation due to genetic drift at the third (variable) codon bases or in repeated and not-transcribed base sequences. Genetic analysis can therefore be useful for discriminating between very closely related viruses and as such comes before the electrophoretic comparison of polypeptides in the above list.

The information provided by each of these four techniques when applied to baculoviruses will be considered in order to throw light on the interrelationships of the viruses in this group.

Most electrophoretic profiles of restriction enzyme digests of baculovirus genomes show little or no similarity between viruses (Rohrmann et al., 1978; Smith and Summers, 1978). Genomes with 15% or more base substitutions will show less than 10% conserved fragments (Upholt, 1977), so this merely indicates that most baculoviruses are not very closely related. On the other hand some baculoviruses show very high levels of sequence conservation by this method, e.g. several geographical isolates of Mamestra brassicae NPV and Heliothis armigera NPV (Vlak et al., 1981); variants of Pieris spp GV (Crook, 1981 (b)); Autographa californica NPV and the related NPV's of Rachiplusia ou, Trichoplusia ni and Galleria mellonella (Smith and Summers, 1979). Similar results are found by homology studies using hybridisation techniques notably Southern blotting. Vlak et al. (1981) showed little homology ($\leq 10\%$) between a range of sNPV's, mNPV's and GV's either within or between these groups, except in one or two cases where closely related viruses had more than 80% homology. Jewell and Miller (1980) found very low levels of homology ($\leq 1\%$) between five NPV's of which three were multiply enveloped and two singly enveloped.

The conclusion to be drawn from these studies of the viral DNA is that some viruses are very closely related, with high levels of sequence homology, and are probably genetic variants rather than separate viruses. These variants appear capable of genomic recombination on multiple infection (Croizier et al., 1980; Croizier et al., 1981; Summers et al., 1980). NPV's contain multiple genome copies in each I.B. and these can consist of genetic variants. It is necessary to clone individual variants for fine genetic analyses (Lee and Miller, 1978).

Apart from these variant forms most baculoviruses exhibit low levels of sequence homology. An exception to this is the study by Kelly (1977) showing intermediate homology (15-40%) between isolates of Spodoptera spp.

The baculovirus subgroups cannot be distinguished by these methods nor by information on base composition or DNA molecular weight (Harrap and Payne, 1979).

SDS polyacrylamide gel electrophoresis of viral polypeptides often shows variants in parallel with differences in restriction enzyme digest profile, for example in a study of two isolates of M. brassicae NPV (Groner, 1978; Vlak and Groner, 1980). Crook (1981(a)) found that the virus particle polypeptides of Pieris brassicae and P. rapae GV's were almost identical, small differences residing in the viral envelopes. These viruses were clearly distinguishable by restriction enzyme analysis however. The I.B. polypeptide exhibits very small size difference with different viruses (Croizier and Croizier, 1977) which cannot usually be used to discriminate viruses. Discrimination is possible however if a protease is used to partially digest the polypeptide giving specific digestion products (Vlak et al., 1981). No systematic differences between subgroup, viral or I.B. proteins have been found by electrophoretic methods (Payne and Kelly, 1981).

Serological studies are often difficult to interpret due to the variety of techniques for antiserum production and testing which have been used. Hoops and Summers (1978) found that when antiserum was prepared to Trichoplusia ni GV I.B. protein isolated without alkaline protease action, three precipitin lines were produced in gel diffusion tests with the homologous antigen. Two of these lines formed a reaction of identity with Spodoptera frugiperda GV granulin and polyhedrin from several mNPV's and a sNPV. However if protease degraded granulins and polyhedrins were used as antigens with the same antiserum there were fewer precipitin lines and greater specificity. Croizier and Meynadier (1975) also found cross reactions between several lepidopteran NPV's

using immunodiffusion tests with antisera to NPV polyhedra or polyhedrins. Virus particle components usually produce more specific antisera. Thus although the very closely related Pieris brassicae and P. rapae GV's could not be distinguished by immunodiffusion test using virus particle antisera, they could be separated by double antibody sandwich ELISA (Crook, 1981(a)). Harrap et al. (1977) could distinguish three biochemically related NPVs from Spodoptera spp. by immunodiffusion using antisera to nucleocapsids. Similarly McCarthy and Lambiase (1979) showed that sNPV's and mNPV's from plusiine hosts cross reacted extensively in immunodiffusion using polyhedrin antisera but only slightly with nucleocapsid antisera. Pritchett et al. (1979) also found that polyhedrins were antigenically cross-reactive when testing lepidopterous mNPV's and sNPV's, while virus particle antigens were mainly virus specific.

Conversely virus particles from different subgroups can give cross reactions on gel diffusion testing, for example Oryctes virus and NPV virions (Payne et al., 1977(a)). However, polyhedrin did not act as a group antigen when lepidopterous NPV's were compared with Tipula paludosa NPV by immunodiffusion (Guelpa et al., 1977).

Smith and Summers (1981) have adopted a new approach to this problem. By separating baculovirus structural polypeptides by PAGE and then blotting them onto nitrocellulose, a radioimmunoassay can detect which individual polypeptides are antigenically cross reactive to a particular antiserum. Using this technique it was found that mNPV, sNPV and GV I.B. proteins cross react extensively to mNPV polyhedrin antiserum. Using antiserum prepared to purified virions there was considerable cross reaction between viral polypeptides within the mNPV and sNPV subgroup, but much less between the NPV and GV subgroups. The authors mention that homology

studies (in press) support these conclusions. These results agree with the morphological subgrouping among lepidopterous baculoviruses. Recent work by Rohrmann et al. (1981) however, suggests that lepidopterous NPV's may be more closely related to GV's than to dipteran or hymenopteran NPV's. This is based on results of double antibody sandwich radioimmunoassay using antisera to Orgyia pseudotsugata mNPV polyhedrin. The sNPV from the species reacted with almost the same intensity as the mNPV. Pieris brassicae GV showed a weaker reaction but stronger than that of Neodiprion sertifer and Tipula paludosa NPV's which showed very low level reactions.

The serological studies to date have shown that group specific antigens exist within the lepidopterous baculoviruses and these viruses can be distinguished by serological methods from other virus groups (Crook and Payne, 1980). While the lepidopterous NPV and GV subgrouping may have phylogenetic meaning, the position of hymenopterous and dipterous NPV's and the Oryctes virus subgroup is not clear.

Only a limited number of studies of amino acid composition have been done on baculoviruses. Summers and Smith (1976) found that the N-terminal amino acid from I.B. protein of Autographa californica mNPV and Trichoplusia ni sNPV was glutamic acid while that from Trichoplusia ni and Spodoptera frugiperda GV's was asparagine. A subgroup difference may exist here. Rohrmann et al. (1981) have done limited sequencing studies on I.B. proteins from a range of baculoviruses. The results suggest that Bombyx mori and O. pseudotsugata mNPV's are closely related to each other but slightly less so to O. pseudotsugata sNPV. P. brassicae GV, N. Sertifer NPV and T. paludosa NPV are distinct from each other and from the lepidopteran NPV's.

It is concluded that lepidopteran NPV's are a distinct group, the sNPV's and mNPV's being closely related. GV's are similar but distinct from these. Other baculoviruses including NPV's of Diptera and Hymenoptera should perhaps be grouped separately from those of Lepidoptera but here relationships are unclear (Payne and Kelly, 1981).

b) Cytoplasmic polyhedrosis viruses

These viruses form a genus within the family Reoviridae (Matthews, 1979). The naked icosahedral virus particle is 50-60 nm in diameter and contains a ds RNA genome of 10 discrete segments with molecular weight totalling about $13 - 16 \times 10^6$ (Harrap and Payne, 1979). On the basis of the electrophoretic profile of the ds RNA segments from lepidopteran CPV's it has been possible to distinguish 12 virus 'types' (Payne and Rivers, 1976). The virus particle is composed of 3-6 polypeptides of molecular weight $30 - 160 \times 10^3$ and their electrophoretic profile confirms the type classification (Payne and Rivers, 1976).

CPV's produce polyhedral I.B.'s ranging in diameter from less than $1 \mu\text{m}$ to $10 \mu\text{m}$ and there is no polyhedral membrane present (Payne, 1981(b)). The matrix protein has a molecular weight of $25 - 30 \times 10^3$ and is virus specified (Mertens, 1979). This polypeptide and the virus particle polypeptides appear to be primary gene products of individual RNA segments (Payne and Rivers, 1976; Mertens, 1979).

The different virus types are antigenically unrelated in terms of matrix protein and virus particle polypeptides but cross reactions between closely-related viruses within one type can occur (Payne et al., 1980). The relationships between CPV's from different host orders or different lepidopteran CPV 'types' are unknown.

c) Entomopoxviruses

These are members of one genus in the family Poxviridae and can be divided into three subgenera on the basis of virus particle morphology (Matthews, 1979; Kurstak and Garzon, 1977). Harrap and Payne (1979) summarise the limited amount of information available on the biochemistry of this group. The genome is composed of linear ds DNA with molecular weight $130 - 200 \times 10^6$ and up to 40 polypeptides may be present in a virus particle.

A recent study of DNA homology has shown little relationship between viruses from the three different subgenera, whereas Amsacta moorei and Euxoa auxiliaris EPV's from subgenus B have about 50% homology. A. moorei EPV DNA showed no homology with Vaccinia virus DNA (Langridge, 1981).

(3) Inclusion Body Design

Most studies on occluded viruses mention the approximate size of the I.B. but rarely is any further analysis undertaken. Where electron microscopic analysis of thin sections has been done it has been possible to estimate the number of virus particles per I.B. where this is very small, in addition to the number of nucleocapsids per virus particle for multiply enveloped viruses. No attempts have been made to calculate the number of virus particles in large I.B.'s from this data. It has become clear, however, that a wide range of I.B. designs are found in baculoviruses and CPV's. EPV's have received little attention.

Anopheline mosquitoes usually have small CPV I.B.'s occluding one virus particle. For example Anopheles quadrimaculatus CPV has spherical or cuboidal I.B.'s of about 150 nm diameter (Anthony et al., 1973).

These might be considered analagous to lepidopteran GV's which usually have one virus particle in each I.B. Here the I.B. is termed a 'capsule'. Both GV's and anopheline CPV's exhibit occasional 'aberrant' I.B.'s containing several virus particles. A. quadrimaculatus CPV produces some I.B.'s with up to nine virus particles (Anthony et al., 1973). Although the authors ascribe this to coalescence of smaller I.B.'s, they have no real evidence for this. It is more likely that the large I.B. is produced de novo and randomly occludes several virus particles. Plodia interpunctella GV also produces aggregations of this sort (Arnott and Smith, 1968). Up to five virus particles per capsule have been found in Laconobia oleracea GV (Meynadier et al., 1969) while two are common (Crook et al., 1982), and Pinnock and Hess (1978) found two virus particles in some capsules of Archips argyrospila GV. Granulosis viruses also sometimes exhibit multiply enveloped virus particles, with up to nine nucleocapsids per virus particle in P. interpunctella GV (Arnott and Smith, 1968); four in A. argyrospila GV (Pinnock and Hess, 1978) and two in Spodoptera frugiperda GV (Bergold, 1963). Other GV's show no apparent morphological aberrations of this type. No evidence of this was found in a study of Pieris brassicae GV, for example (Brown et al., 1977).

Culicine mosquito CPV I.B.'s are usually larger than those of anophelines and several virus particles are occluded as the norm. Aedes cantator CPV I.B.'s range in diameter from 0.5 μ m to 3 μ m (Andreadis, 1981) while those of Aedes sollicitans and Culex salinarius are 0.1 μ m to 1 μ m (Clark et al., 1969). The I.B.'s of these viruses are analagous to those of NPV in design and in both cases any one virus produces inclusion bodies with a range of sizes known as 'polyhedra'. The size range may be caused by polyhedra from different infected cells differing in size, (x)

presumably as a result of the number of polyhedral initiation sites within each cell. Any one cell produces polyhedra of similar size (Vago and Cayrol, 1955; Huber, pers. comm.). It is not clear therefore to what extent polyhedral size is a virus specified feature and how it may be modified by host cell type. It does seem that polyhedral shape is virus determined as it is retained on passage both with CPV's (Payne and Harrap, 1977) and NPV's (Harrap and Payne, 1979). While usually being pentagonal, hexagonal or globular in outline some NPV I.B.'s are more distinctive, notably the cubic I.B.'s of Autographa californica NPV (Vail et al., 1973) and the pyramidal I.B.'s of Panaeus duorarum NPV (Couch, 1974). Such morphological features can be of diagnostic value (Jurkovicova, 1979(a); Vail et al., 1973). It seems that I.B. size is under less rigid viral control so that a virus isolate exhibits a characteristic range of sizes (Jurkovicova, 1979(b)).

The number of virus particles occluded varies widely among the larger polyhedral viruses, appearing to depend to some degree on polyhedral size. With NPV's both Morgan et al. (1956) and Bergold (1963) have suggested that the virus particles are randomly embedded and they do not appear to disrupt the crystalline lattice. More recent work confirms this except for one study on Orgyia pseudotsugata sNPV when Hughes and Addison (1970) found some polyhedra with regular arrangement of virus particles.

Some CPV's contain the largest numbers of occluded virus particles, with an estimate of up to 10,000 per I.B. in Danaus plexippus CPV (Arnott et al., 1968). Martignoni (Pers. Comm.) found up to 265 virus particles per section of Autographa gamma CPV I.B. By partial dissolution of NPV polyhedra it is possible to retain the virus particles within the

polyhedral membrane and count them using electron microscopical examination of negatively stained preparations. This technique is most suitable for sNPV and small mNPV. Using this method Gregory et al. (1969) estimated a mean of 26.4 virions per I.B. with Heliothis zea sNPV, the results ranging from 15 to 38. Lipa et al. (1971) observed up to 40 virus particles per polyhedron of Agrotis segetum mNPV while Smirnoff and Ackermann (1977) estimated up to 80 in Thymelicus lineola sNPV polyhedra.

With mNPV's the number of nucleocapsids per I.B. may be many times greater than the number of virus particles due to multiple envelopment. The distribution of nucleocapsids per virus particle can be analysed by isopycnic centrifugation of alkali liberated virions on sucrose gradients. However, this method often produces bands of viral degradation products (Cibulsky et al., 1977; Jurkovicova, 1979(b)). Electron microscopy of sectioned polyhedra is more suitable. The mode and distribution of enveloped forms varies with different viruses. Orgyia pseudotsugata NPV usually has 2 to 15 nucleocapsids per bundle but up to 28 can be found (Hughes and Addison, 1970). Bergold (1963) studied two Lymantria spp. NPV's and one NPV from Spodoptera frugiperda; again few singly enveloped nucleocapsids were found and up to 19 were enveloped together. A detailed study of these packing arrangements was made by Kawamoto and Asayama (1975) in Euproctis similis NPV. Certain arrangements were more common presumably for reasons of stability. Hexagonal organisation of transversely sectioned virus particles was found most often, with up to 39 nucleocapsids per bundle. Not all NPV's have such large virus bundles. Anticarsia gemmatalis NPV has small polyhedra (1.2-1.5 μ m) with 43% of virus particles singly enveloped while only 2% have more than 5 nucleocapsids (Carner and Turnipseed, 1977). Similarly Vlak and Groner (1980) found 1-8 nucleocapsids per virus particle in two isolates of

Mamestra brassicae NPV and Lipa et al. (1971) observed 4-8 nucleocapsids per bundle in A. segetum NPV. Lymantria dispar NPV has 2-7 nucleocapsids per virus particle (Morgan et al., 1956).

It is probable that these arrangements are stable during virus passage. Tomkins et al. (1980) found only very small changes in the number of virus particles per NPV I.B. section and the number of nucleocapsids per virus particle when Heliothis armigera and Rachiplusia ou mNPV's were passaged several times in alternative hosts.

Kawamoto and Asayama (1975) suggested that the limiting factor for virus particle size might be the envelope size into which the nucleocapsids pack. If so it would indicate a mechanism for viral control of this feature.

By combining data on number of virus particles per I.B. and size of virus bundles it would be possible to calculate the number of viral genome copies per I.B. for mNPV's. Unfortunately both pieces of data are rarely available for one virus. It is clear that mNPV's may have several hundred nucleocapsids in each I.B. Hughes (1979) estimates that Orgyia pseudotsugata mNPV has about 1600 while O. pseudotsugata sNPV has about 670.

It can be concluded that as a result of alternative packing arrangements, CPV and baculovirus I.B.'s can enclose one to several thousand genome copies. Similar data for EPV's is lacking. Milner and Beaton (1979) estimated that Oncopera alboguttata EPV contains about 80 virus particles per I.B. Kurstak and Garzon (1977), however, claim that the number of virus particles occluded by each spheroid (EPV I.B.) varies in different tissues and in different hosts.

(4) Pathology of Infection by Occluded Viruses

The major pathway for infection by occluded viruses appears to be via feeding on material contaminated by I.B.'s. The I.B.'s dissolve in the insect gut to release virus particles. These infect the midgut initially, (solely in the case of CPV's and some baculoviruses) but also a range of other tissues in infections by EPV's and lepidopteran baculoviruses. The replication cycle is completed with the production of more occluded virus which may be released with the frass by CPV infected larvae, or at death (which is associated with rapid tissue breakdown) in baculovirus infections of Lepidoptera. These processes are considered in detail below, concentrating on the initial stages most pertinent to this study.

a) Inclusion body dissolution

Most studies of dissolution have examined the process in vitro. It is found that most baculoviruses and CPV's dissolve readily in carbonate buffer of moderately high pH to produce particles infective by haemocoelic injection (Paschke and Summers, 1975; Payne, 1981(b)). For example Pieris brassicae GV dissolves rapidly in 0.1 M carbonate buffer at pH 9.8 but not at pH 8.95 (Ripa, 1978). Egawa and Summers (1972) demonstrated that the process had three phases using Trichoplusia ni GV. These are the lag, exponential and slow phases of dissolution. It was suggested that the first is caused by the presence of a protective surface structure which ruptures rather than dissolving. The last phase is due to the presence of resistant I.B.'s in the population.

Tipula paludosa NPV shares with the entomopoxviruses the necessity for reducing conditions to dissolve I.B.'s in vitro. Bergoin and Guelpe (1977) used 0.125 M sodium thioglycollate with 0.125 M sodium bicarbonate

at pH 10.5 to release intact virus particles by dissolution of T. paludosa NPV I.B.'s. Oncopera alboguttata EPV I.B.'s require carbonate/thioglycollate at pH 12 for dissolution (Milner and Beaton, 1979). It is probable that in these cases disulphide bridges are involved in the matrix structure (Bergoin and Guelpa, 1977), in contrast with weaker ionic bonds for most baculovirus inclusions (Egawa and Summers, 1972). Early suggestions that the matrix of lepidopterous baculoviruses might be held together by a siliceous framework (Faust and Adams, 1966), have now been largely discounted (Padhi and Chase, 1976).

The situation in vivo is more complicated. Paschke and Summers (1975) suggested that crystal dissolution does not need carbonate ions or high pH in vivo. Faust and Adams (1976) found that dissolution of three NPV's in Bombyx mori midgut juice occurred at a slower rate after heat treatment of this juice, suggesting some role for enzymes in the dissolution process. Gut juice dialysed against borate buffer pH 8.5 retained proteolytic activity but could not dissolve polyhedra, so pH is also important.

Milner and Beaton (1979) note that the gut of O. alboguttata is probably not at the high pH required for dissolution in vitro (see above) so presumably enzyme action is also involved here. On the other hand the midgut pH of Pieris brassicae larvae is certainly sufficient to dissolve its granulosis virus (Ripa, 1978), and three species of Aedes mosquitoes have gut pH high enough to dissolve Aedes NPV (Stiles and Paschke, 1980). Broome and Coons (1978) speculate that enzymic degradation of the heteropolysaccharide polyhedral envelope of Heliothis virescens NPV may be necessary before action of protease on the matrix.

Where the dissolution process has been observed in vivo it appears to be a rapid process both for CFV and baculovirus. A CFV from Noctua pronuba entered the midgut 5-6 minutes after feeding and then dissolved rapidly (Vago et al., 1959). Heliothis zea NPV I.B.'s dissolved almost completely within one hour in H. zea larvae (Granados, 1978(a)), while Autographa californica NPV dissolved within 15 minutes post-infection in fourth instar larvae of Trichoplusia ni (Granados and Lawler, 1981). Paschke and Summers (1975) show that in many cases baculovirus dissolution is not a specific process in infection but occurs in insects other than the natural host.

b) Virus survival in the gut lumen

The midgut epithelium seems to be the main site of initial penetration for baculoviruses, CFV's and EFV's (Paschke and Summers, 1975). Limitations to infection at this point include degradation of the free virus before reaching the attachment site, a process exacerbated by barriers to free movement of the virus, notably the peritrophic membrane (Section 4c). In addition, attachment to the cell membrane presumably involves specific receptors and their presence and density will be important in determining the level of infectivity (Section 4d).

Virus degradation in the gut lumen is a common phenomenon. While Granados (1978(a)) failed to observe degradation of Heliothis zea NPV in H. zea larval midgut, Langridge (reported in Granados, 1980) found almost 100% inactivation when this virus was exposed to H. zea gut juice for 60 minutes at 26°C in vitro, as measured by intrahaemocoelic infection potential. Summers (reported in Paschke and Summers, 1975) estimated that at least 50-60% of Trichoplusia ni GV and Spodoptera frugiperda GV are degraded in the lumen of larvae of the homologous host. However, in these

cases sufficient virus survives to infect the larvae. When the non-occluded mosquito iridescent virus was studied in Aedes taeniorhynchus larval gut it was found to be degraded shortly after entering the midgut (Stoltz and Summers, 1971). S. frugiperda GV was used as a marker and although it dissolved completely, no degradation of virus particles was noted. It is possible however that loss of infectivity may be produced without gross structural modifications of the sort amenable to electron microscopical analysis.

From these and similar studies it appears that the gut is an environment hostile to virus survival. Ripa (1978) has proposed a general positive relationship between gut pH and oral LD50 for many virus host interactions. Whether this is a causal relationship was not established.

It is possible that the I.B. provides some protection to virus by reducing its exposure to gut juice, and perhaps acting as a buffer or protease block after dissolution. Several workers have shown that CPV alkali liberated virus particles may have little or no infectivity per os (Payne and Harrap, 1977). Watanabe (1966(a)) found that Bombyx mori CPV virus particles were much more infective when injected than when administered orally. B. mori strains showing a variation in susceptibility to oral infection did not exhibit this variability on injection, suggesting an inhibitory mechanism in the gut. The pattern is less clear with baculoviruses. Ripa (1978) dissolved Pieris brassicae GV with buffers and gut juice at pH 9.9-10.0 for 1 minute and thereby reduced the oral infectivity to second instar larvae to 0.01% of untreated I.B.'s. Stairs (1980) found the same level of reduction of oral infectivity to third instar larvae of Galleria mellonella when virus particles were released from G. mellonella NPV I.B.'s by treatment with 0.007 M sodium carbonate

for one hour at 24°C. Arif and Brown (1975) on the other hand, produced Choristoneura fumiferana mNPV virus particles which were infective to sixth instar larvae per os. In this case the LD50 value given was 1 µg of virus particles. This would be roughly equivalent to 10⁷ PIB (Chapter 4) which is in the usual range of PIB numbers required to infect final instar larvae (Section 6(a)) suggesting that little degradation occurred. The data of Volkmann and Summers (1977) is more easily quantified. They used a very careful dissolution method and found that the oral LD50 of Autographa californica NPV to Trichoplusia ni mid-fourth instar larvae was 2.3 x 10⁴ genome equivalents of released virus particles or 120 undissolved whole polyhedra. They estimate 36 genome equivalents per I.B., so 120 I.B.'s corresponds to 4.3 x 10³ genomes. This would make undissolved I.B.'s only 5 fold more infective than released virions. In fact 36 seems a low estimate for genomes/I.B. (see Chapter 4) in which case the difference in infectivity is smaller still. It is possible therefore that many studies have inactivated virus particles during artificial dissolution and that degradation in the larval gut is not important. Vail et al. (1979) dissolved A. californica NPV with larval gut juice obtained from Estigmene acrea and found that only 0.0025-0.0050 polyhedral equivalents (approximately one virus particle) were required to infect T. ni tissue culture cells. The infectivity started to fall after 8 minutes of digestion. This suggests that virus particles can survive degradation for a significant period of time when I.B.'s are dissolved in gut juice. The evidence points to the limited importance of I.B.'s as protective structures in the larval gut. This view receives indirect support from the fact that many non-occluded insect viruses also infect mainly by the oral route (Scotti et al., 1981) and yet have developed no analogous 'protective' structure.

c) The peritrophic membrane as a barrier to infection

Attachment of all insect viruses studied so far occurs at the midgut epithelium. This is the endodermal part of the insect gut and as such is not lost at the larval moult. It is the major site for absorption (Smith, 1968). Food entering the midgut is contained in a protein/chitin membrane secreted by the midgut. This is the peritrophic membrane (PM) and its function is poorly understood (Richards and Richards, 1977).

It is often suggested that the PM may limit passage of virus particles to the midgut epithelium (Paschke and Summers, 1975). However the real effect of this has been difficult to quantify. The membrane often appears continuous with no pores, for example in Aedes taeniorhynchus (Stoltz and Summers, 1971). In healthy Orygia pseudotsugata larvae three layers could be discerned of which the inner was smooth and free of pores (Brandt et al., 1978). However Adang and Spence (1980) studying the same species noted that there were pores of two sizes the larger being up to 660 nm through which baculovirus virions could pass slowly. The PM does appear to act as a barrier to passage of iridescent virus and baculovirus virions in A. taeniorhynchus larval gut (Paschke and Summers, 1975). In Apis mellifera larvae, sacbrood virus particles aggregated around the membrane preventing free movement (Mussen and Furgala, 1977). Orihel (1975) considers that the PM may generally limit the degree of infection of invertebrate vectors by parasites.

On the other hand, some studies with baculoviruses suggest the PM is of limited importance in this context. Tanada et al. (1975) showed that small polyhedra and virus particles of Pseudaletia unipuncta NPV could penetrate the PM of this species. Both GV and NPV virus particles

readily penetrated the membrane in Trichoplusia ni and Spodoptera frugiperda larvae (Stoltz and Summers, 1971).

It seems that the effectiveness of the FM as a barrier to infection depends on the species involved, perhaps being less important in lepidopterous larvae than dipterous larvae. The origin of the membrane differs in these two orders, being secreted as a series of layers by the whole midgut in Lepidoptera but as a homogenous tube produced by anterior gut cells in Diptera (Wigglesworth, 1965). This may explain the differences in penetrability which are found.

To summarise the data on the gut environment as a defence to infection, it seems that in most cases and notably baculovirus infections of Lepidoptera, a large proportion of virus particles can survive. In some other hosts and viruses for example iridescent virus infections of Diptera, a severe reduction in infection potential can occur at this stage. In these examples there is indeed a 'gut barrier' in the sense discussed by Tinsley (1975).

d) Attachment and penetration

Little is known about the specific receptors for attachment of insect viruses. That this attachment stage may be a limiting factor in infection is indicated by the work of Tanada and co-workers. A protein isolated from Pseudaletia unipuncta GV I.B.'s increases the infectivity of P. unipuncta NPV and other baculoviruses (Tanada and Hara, 1975). This 'synergistic factor' has an affinity for the viral envelope (Yamamoto and Tanada, 1980) and appears to act at the microvillar membrane (Tanada et al., 1980) resulting in increased uptake of nucleocapsids (Tanada et al., 1975).

Uptake of viruses may occur by several mechanisms. The CPV's are taken up by viropexis, probably with no disruption of the virus particle which would inactivate the virus associated RNA transcriptase (Payne, 1981(b)). The enveloped baculoviruses and poxviruses are usually taken up by membrane fusion (Summers, 1971; Kawani'shi et al., 1972; Granados, 1973; Paschke and Summers, 1975), although viropexis may sometimes occur (Granados, 1978(a)). Direct invasion into the haemocoel along columnar cell boundaries has been suggested by Tanada and Leutenegger (1970), but is probably not commonplace (Granados, 1980).

Fusion of baculoviruses occurs soon after reaching the midgut (Granados, 1978(b)) and in the case of multiply enveloped virus particles the nucleocapsids remain associated while in the microvillus and separate on reaching its base (Granados and Lawler, 1981). The mechanism of movement of nucleocapsids within the cytoplasm is unknown.

e) Pathways of replication

(i) Baculoviruses. This group exhibits a variety of replication sites both at the tissue and cell level. Lepidopteran infections usually start at the midgut, but mature polyhedra are rarely produced here, being found in a variety of other tissues secondarily infected by virus particles produced in the initial midgut infection cycle (Harrap and Robertson, 1968; Adams et al., 1977). Hymenopterous NPV replication is usually limited to the midgut (Harrap and Payne, 1979). Penaeus duorarum NPV infects the hepatopancreas and midgut of its crustacean host and, unlike most insect baculoviruses, it does not usually cause death (Couch, 1974).

Granados (1978a) summarises the literature on baculovirus replication in Lepidoptera. Trichoplusia ni GV uncoats at the nuclear envelope while

Heliothis zea and Autographa californica NPV's uncoat within the nucleus. The first cycle of infection is complete by 8-12 hours post infection and is completed as the virus becomes enveloped by budding through the plasma membrane into the haemocoel. The membrane-budded virus rapidly infects the other tissues where I.B. formation starts as early as 18-22 hours post infection. In granulosis virus infections the nuclear membrane breaks down during the infection cycle whereas with nuclear polyhedrosis viruses it remains intact until the completion of polyhedra formation. Whether this is a true difference between the subgroups, or just a reflection of the few studies which have been done, remains to be seen.

The membrane-budded virus has distinctly different properties from the occluded virus (Summers, 1977) which produces its envelope de novo in the nucleus (Adams et al., 1977). Membrane-budded virus is much more infective to cultured cells (Volkman et al., 1976) and intrahaemocoelically to larvae (Volkman and Summers, 1977). However, it has much lower oral infectivity than alkali-liberated occluded virus particles (Volkman and Summers, 1977; Stairs, 1980). It is reasonable to suggest that occluded virus is adapted to optimally infect midgut columnar epithelial cells and perhaps to resist the gut conditions found there (Kawarabata and Aratake, 1978). The non-occluded virus produced in the first replication cycle is efficient at infecting a range of other tissues. This initial multiplication step followed by rapid colonisation of other susceptible tissues gives synchrony to the development of inclusion bodies. The gross pathology in lepidopterous larvae involves sudden development of symptoms and death several days after infection. Before the final stage there are often few symptoms. Lysis of cells in many tissues often including the hypodermis results in liquefaction of the cadaver and rapid spreading of

the I.B.'s. The more limited gut infection in Hymenoptera does not produce such sudden changes in symptomatology.

(ii) Cytoplasmic polyhedrosis viruses. Payne (1981(b)) has recently reviewed the pathology of these infections. They are always limited to the midgut in Lepidoptera although there are reports of fat body being involved in one dipteran host. The gross pathology is variable but usually involves reduction or cessation of feeding with related slow growth rate. Death may occur after variable lengths of time probably dependent on the dose received. Infected individuals may pupate and emerge as adults, but often these stages are deformed (Harrap and Payne, 1979). In Choristoneura fumiferana and Malacosoma disstria the infection has progressively less effect as the larvae age although the infectivity does not decrease much (Bird, 1969). Andreadis (1981) notes that CPV infections of mosquitoes usually involve a limited infection which is not fatal and produces few symptoms. However Aedes cantator dies when infected with its CPV.

The midgut epithelium is not infected synchronously. As the disease progresses both mature polyhedra and virus particle are released into the lumen and the latter infect further cells (Payne, 1981(b)). A reduction in gut pH associated with the infection allows release of a continuous stream of infective polyhedra (Vago et al., 1959). This contrasts with the sudden output of inoculum at death in baculovirus infections (Section e(i)).

f) Latency

The possibility that occluded insect pathogenic viruses are able to persist in the host cell in an inapparent form (without causing overt infection) has often been suggested in the literature. The phenomenon of

latency is well known in bacterial and animal viruses such as the temperate phages and Herpes^{ad}viridae. If such persistence is possible this could mean that the overt infection is of little importance to viral survival. In that case it would be difficult to argue that the inclusion body is under strong selective pressure to maximise survival and infectivity. A close study of the literature shows that most workers who claim to have observed an apparent infection induced from a latent source have probably been dealing with contaminated insect stocks or virus inocula. For example Longworth and Cunningham (1968) found that when Aglais urticae and Lymantria (Porthetria) dispar were fed the NPV of the other species they died of their homologous NPV. An examination of their data shows moderate numbers of NPV deaths in control larvae as well as death by CPV and other pathogens in all tests. These levels of contamination prevent serious consideration of the authors' conclusions. Similarly Jurkovicova (1979(a)) studied Adoxophyes orana and Mamestra brassicae NPV's and found that apparent cross-transmission to the alternative host resulted in production of that host's homologous virus. He suggested that there had been stimulation of a latent infection. Again, however, there are virus deaths in control insects and the total mortality in tested insects was only about 10% despite high inoculum concentration.

McKinley et al. (1981) in a superficially convincing paper, show that Spodoptera littoralis larvae infected with Heliothis armigera NPV always produced S. littoralis NPV as the progeny virus. The LD50 for this response was assayed and the necessary inoculum contamination level which would be required to produce this LD50 was calculated. The result appeared to vary by more than 100 fold between assays using the same inoculum which would make contamination an unlikely hypothesis. Recalculation of the results presented in fact shows that the necessary contamination level

varies only 10 fold which is the level of variation often found in large series of assays. In another experiment the authors show that the LC50 ratio between H. armigera and S. littoralis NPV for different stocks of S. littoralis is different. Rather than being due to different inherent levels of latent virus this could be explained by a difference in relative feeding rate on the high virus protein levels in the H. armigera LC50 tests.

Experiments of this sort have been carried out with CPV's and here the contamination problems can be more difficult (Payne, 1981(b)). The low-level chronic infections often exhibited by CPV's make it too easy to introduce contaminated adults from the field into the laboratory stock. The wider host range of these viruses (see Section 5) together with slow development of infection increases the chance of mixed infections and therefore of contaminated inocula. In fact the major interest of the studies described above, where progeny virus has been identified as different from inoculum, is to cast doubts on all cross-transmission literature where this identification has not been done.

To demonstrate latency conclusively it would be necessary to identify the virus in its latent state and study its reactivation. This work has not been done and it must be concluded that there is no good evidence for latency of occluded viruses. Recently a persistent infection of Heliothis zea cells by a baculovirus-like particle has been observed in vitro (Granados et al., 1978; Kelly et al., 1981). This appears overtly at low levels and so is not in the 'latent' state, but it indicates that low level persistent infections of baculoviruses may exist.

(5) Host Range

The literature on virus host range is often difficult to interpret as identification of inoculum and progeny viruses has not been carried out. Usually, there has been no precise dosing of hosts making comparison of results impossible and this is compounded by the arbitrary choice of insect species tested.

Ignoffo (1968), reviewing specificity of occluded insect viruses, recognised these problems but decided to accept all data in the literature at face value. He concluded that granulosis viruses were the most specific group tested, with only 10% of cross-infection tests being successful. There was only one example of a GV infecting a species from a different family. This was the transmission of an unknown cutworm GV to Pieris rapae (Smith et al., 1964). The virus could not be transmitted to six cutworm species and it seems probable that the P. rapae larvae died of contaminating P. rapae GV, a phenomenon found once in the present study (Chapter 5).

Nuclear polyhedrosis viruses of Lepidoptera had a wider host range with 30% success in cross-transmission tests. Twenty per cent of tests to hosts from alternative families were successful and there were three reports of cross-transmission to non-lepidopterans, notably the neuropterans Chrysopa perla and Hemerobius stigma which were susceptible to Lymantria dispar NPV.

CPV's had the widest host range with 65% successful cross-transmissions and 46% of trans-familial tests succeeding. Vanessa io CPV infected the same two neuropteran hosts mentioned above and these were the only trans-ordinal reports. The pattern established by Ignoffo's review has generally been confirmed by more recent work.

Huber (1978) found only limited cross-transmission of Cydia (Laspeyresia) pomonella GV and then only to members of the same subfamily. The GV of another tortricid Adoxophyes orana infects A. fasciata but not five tortricids from other genera (Yamada and Oho, 1976). Philip and Jacob (1979) noted that the GV of an arctiid Pericallia ricini is species-specific. A slightly wider host range was found by Tanada and Omi (1974) in a study of unidentified GV's from four noctuid species. These each infected all four species but did not infect the pierid Colias eurytheme.

It is interesting to look at NPV cross-transmission data in the light of recent characterisation studies mentioned in Section 2(a). Autographa californica NPV has the widest established host range of all the baculoviruses studied. A fairly complete list of its hosts is given by Capinera and Kanost (1979) which includes members of the families Noctuidae; Geometridae; Saturniidae; Arctiidae; Lasiocampidae; Lyonetidae; Yponomeutidae; Pyralidae. NPV's have been isolated from Trichoplusia ni, Rachiplusia ou and Galleria mellonella which show a high degree of homology with A. californica NPV (Smith and Summers, 1979). Several studies on the T. ni NPV suggest it has a similar host range (Tanada and Omi, 1974; Reichelderfer and Benton, 1974; Beegle and Oatman, 1975; Harper, 1976). It seems that these mNPV's form a complex of variants with wide host range among the Lepidoptera. Presumably the variants have developed as a result of the wide geographical and ecological distribution this wide host range allows.

Similar complexes may occur in the old world. For example the NPV of Mamestra brassicae infects many hosts within the family Noctuidae (Groner, 1978; Ignoffo and Garcia, 1979; Vlak and Groner, 1980), but not

other families (Burgerjon, 1973). Geographical variants of the virus are also found, including the mNPV of Heliothis armigera (Vlak et al., 1981). Again there seems to be an association between the ability to infect a range of hosts covering a large area and variety of habitats, and the existence of several variants of the virus. More characterisation work on other viruses will be necessary to support this pattern.

sNPV's can be difficult to grow in cell culture (Volkman, 1978; Kelly et al., 1981), although unlike GV's it is possible to do this (Granados, 1976). This may be associated with a smaller in vivo host range of these viruses. Tests by several workers have shown that Heliothis sp. sNPV only infects members of this genus (Ignoffo and Couch, 1981). However some sNPV's have wider host ranges. Pseudoplusia includens sNPV infected two other genera of noctuids, and Syngrapha selecta sNPV four genera in a study by Harper (1976). Watanabe et al. (1975) infected the pyralid Chilo suppressalis with Bombyx mori (Noctuidae) sNPV by haemolymph injection.

Lepidopterous baculoviruses usually infect a very limited range of species within the same family and occasionally in other families. Where wide ranging tests of infectivity to other arthropod classes or non-arthropods have been carried out, there have been no successful cross-infections (Ignoffo, 1968; Burges et al., 1980). Non-lepidopteran baculoviruses appear to be generally genus specific (Burges et al., 1980).

The CPV's exhibit transmission between families quite frequently. Tanada and Omi (1974) infected four noctuids with a CFV isolated from the pierid Colias eurytheme. The reciprocal transmissions were also successful, although the same virus may have been involved as there was no identification. Several workers have found that Bombyx mori CFV can

infect members of other families (Yamaguchi, 1979; Tanaka, 1971).

Similarly a CPV isolated from the nymphalid Inachis io infected members of the Pieridae and Noctuidae (Wilson, 1981).

The limited data on EPV's suggest they are quite specific.

Euxoa auxiliaris EPV typhsin treated virions infected the homologous host by injection but not four other noctuids, two pyralids or an arctiid (McCarthy et al., 1975). The EPV of the melolonthine scarab beetle Dermolepida albohirtum did not infect five other species of this subfamily (Goodwin and Roberts, 1975).

Although CPV's have a slightly wider host range than the other occluded viruses it is still much smaller than that of many of the non-occluded insect viruses. For example Chilo iridescent virus appears to infect a centipede (Chilopoda) and a pill millepede (Crustacea) (Ohba and Aizawa, 1979). Cricket paralysis virus can infect Drosophila melanogaster (Diptera), Galleria mellonella (Lepidoptera) and Gryllus bimaculatus (Orthoptera) by injection (Plus et al., 1978). Compared with these examples the occluded viruses must be seen as having a limited host range. The host range of these viruses in cell culture may differ from that in vivo, but this is not the concern of the present study. The subject is reviewed by Granados (1976) and Knudson (1978).

(6) Oral Infectivity

a) Dose studies

Two classes of method can be distinguished which have been used for studies on dose-related infection:-

(i) Concentration assays. These estimate the median lethal concentration (LC50) or the median infective concentration (IC50).

Here the virus is mixed with or smeared on the larval food. The absolute dose acquired by the test insect is a function of feeding rate and behaviour as well as virus concentration. Results are only comparable when the same stage of one species is dosed under identical conditions.

(ii) Discrete dose assays. These estimate the median lethal dose (LD50) or median infective dose (ID50). Here an accurately measured dose is given to each insect over a relatively short time period and different stages or species can be compared.

Most of the literature concerns baculovirus infection of lepidopterous larvae. It is usual to consider infectivity in terms of the number of I.B.'s required rather than the quantity of virus particles despite the large variation in I.B. size (Section 3). Comparative infectivity of alkali released virus particles has been considered in Section 4(b).

Few I.B.'s of any virus may be required to cause death particularly in young larvae. McKinley (1979) calculated an LD50 of less than one polyhedron for one to three day old Spodoptera littoralis larvae infected with the homologous NPV. Similarly the NPV of Heliothis zea has an LD50 of nine polyhedra for three day old larvae (Allen and Ignoffo, 1969). The LD50 for first instar Cydia pomonella with its GV was 5 capsules (Sheppard and Stairs, 1977).

CPV infections do not always lead to death but are often chronic in nature (Payne, 1981b). Orgyia (Hemerocampa) pseudotsugata fourth instar larvae required eight polyhedra of the homologous CPV as the LD50 (Martignoni et al., 1969). However the LC50 for the CPV infection of neonate Pectinophora gossypiella was about a thousand fold greater than the IC50 (Bell and Kanavel, 1976).

Within a population at the same stage of development there is a (x)
normal distribution of susceptibility to virus infection and this is the
major constituent of the dosage-response slope. The slope is typically
low for virus infections, being approximately 0.8-2.5 due to the
independent action of the individual pathogens (Burgess and Thomson,
1971). It can be increased to greater than 3.0 by artificial selection
of a homogenous test population (Evans, 1981(a)). The significance of this
variation in nature is that some individuals are susceptible to a
fraction of the LD50 dose and so can be important in initiation of an
epizootic. For example Sheppard and Stairs (1977) assayed C. pomonella
GV in fifth instar larvae and found that the LD50, at 49 capsules, was
ten fold greater than that for first instars. The slope of the response
was much shallower for the older insects however, so that both for first
and fifth instar larvae 25% mortality would follow from ingestion of one
capsule.

In most studies it has been found that the LD50 increases rapidly
with age, this increase being proportional to the weight of the insects
tested. H. zea NPV LD50 for eight-day old larvae was 250 fold greater
than for three-day old larvae and this was mainly explained by increase
in body weight (Allen and Ignoffo, 1969). Similarly, Evans (1981(a)) found
that 90% of the observed variation in LD50 to different M. brassicae
instars of the homologous NPV was related to weight. A similar
conclusion was reached by Payne et al. (1981) for the infection of
Pieris rapae with P. brassicae GV and by Boucias and Nordin (1979) with
the CPV infection of Hyphantria cunea. The sawfly Gilpinia hercyniae
also shows decreasing susceptibility with age, in this case the fifth
instar is ten fold less susceptible than the first instar (Entwistle,
Adams and Evans unpublished; reported in Evans and Harrap, 1982).

Changes in susceptibility may not necessarily, however, be directly proportional to weight. NPV and GV infections of H. cunea were studied by Boucias and Nordin (1977(a)), who found that in both cases the fifth instar LD50 was 50-100 fold greater than that for second instars after weight had been accounted for. In the study by Sheppard and Stairs (1977) on C. pomonella GV, the difference in interinstar susceptibility was less than expected on the basis of weight. Bird (1969) showed that the increase in resistance with age was more extreme in NPV than CPV infections of Choristoneura fumiferana and Malacosoma disstria.

In the few cases where LD50 values have been converted into number of virus particles involved it has been apparent that this figure may vary widely. Clearly low LD50 values for capsules of GV infections correspond to similarly low numbers of virus particles. Allen and Ignoffo (1969), calculated that the LD50 of H. zea mNPV for three-day old larvae was 250 virus particles (9 polyhedra). As mNPV's may have hundreds or thousands of nucleocapsids per I.B. (Section 3), at least this many must be required for infection of even the most susceptible larvae.

Where NPV and GV have been isolated from one species the relative infectivity of these viruses shows an interesting pattern. In a study on H. cunea (Boucias and Nordin, 1977(a)), between 150 and 1000 fold more GV capsules than NPV polyhedra were required as the LD50 for second, fourth or fifth instar larvae. As each polyhedra contains 25-30 virus particles each with 2-12 nucleocapsids (Nordin, 1971), it is probable that the number of nucleocapsids is fairly constant for LD50 of both viruses to a particular instar. In the same way Paschke et al., (1968) found that the LD50 for Trichoplusia ni NPV to T. ni fourth instar larvae was 1.23×10^3

polyhedra while the GV LD50 was 7.79×10^{-6} mg capsules. Assuming each capsule is about 0.03 pg (Chapter 4), this corresponds to 2×10^5 GV capsules or a 150 fold greater number of capsules than polyhedra. The only other reported comparison of the infectivity of a GV and NPV to the same species is by Whitlock (1978). Calculation of LC50's from his data, using the same capsules per mg relationship, shows that 100-1000 fold more GV capsules than NPV polyhedra are required for the LC50 to Heliothis armigera seven-day old larvae.

These studies suggest that where a species is infected by homologous NPV and GV infections the GV is much less infective in terms of number of inclusion bodies. Where a GV infection only is known to occur naturally, the virus is often much more infections. For example Cydia pomonella GV (Sheppard and Stairs, 1977) and Pieris spp. GV's in P. rapae (Crook, 1981(b)).

The significance of multiple as opposed to single nucleocapsid envelopment is not clear. Ignoffo et al. (1974(a)) found that T. ni larvae were equally susceptible to T. ni mNPV and sNPV as well as Autographa californica mNPV. Volkman et al., (1976) studied alkali-released virus particles from A. californica mNPV and found that singly and multiply-enveloped virus particles had identical infectivity to T. ni cells. Further studies of these different forms in vivo will be necessary to see if there is a real difference in biological properties.

Where a virus infects several species it is often less infectious for hosts other than that from which it was originally isolated. Diacrisia virginica GV is ten fold less infective than Hyphantria cunea GV for H. cunea second instars (Boucias and Nordin, 1977(a)(b)). The LC50 of Mamestra brassicae NPV for M. brassicae, Plusia gamma and

Trichoplusia ni was similar but 100 fold greater for two Agrotis spp. (Burgerjon, 1973). Payne (1981(a)) however, estimated that the LD50 of Cydia pomonella GV for neonates of the closely related species C. nigricana was only two to three fold greater than for C. pomonella neonates. A GV originally isolated from Pieris brassicae is more infectious to P. rapae (Payne et al., 1981). This virus appears to be one of a set of variants all of which infect P. rapae more successfully than P. brassicae (Crook, 1981(b)) and it is probable that the latter species is not the most frequent natural host of the GV. Its original isolation in P. brassicae may therefore be considered anomalous.

Several studies have examined variation in the infectivity of virus isolates or closely related variants from different geographical areas. Shapiro and Ignoffo (1970) tested 34 isolates of Heliothis spp. NPV and found a 56 fold variation in LC50 to a standard H. zea larval stock. However the identity of the isolates was not checked. When Teakle (1979) also examined a range of Heliothis spp. NPV's from Africa, North America and Australia he found very similar infectivity. This is the more usual result. Brown and Evans (1980) assayed two distinct strains of West European M. brassicae NPV in M. brassicae third instar larvae and found only a three fold variation in infectivity. Similar results were found by Vlak et al. (1981) with isolates of this virus including some from Eastern Europe. The NPV's of Lymantria monacha and L. dispar which are closely-related but distinct by polypeptide analysis and serology, show very similar infectivity for the homologous and heterologous hosts (Zethner et al., 1979). Similarly in an examination of four very closely related isolates of Pieris spp. GV, Crook (1981(b)) found that their infectivity to third instar P. rapae varied only four fold. However the LD50 of these variants for P. brassicae were much higher with greater than

10,000 fold variation. The non-occluded baculovirus isolated from Oryctes rhinoceros exhibits a moderate (seven fold) variation in infectivity between ten isolates from the Philippines and Western Samoa (Zelazny, 1979).

Where susceptibility of insect populations from different geographical areas has been tested, small to moderate variations have usually been found. Two isolated populations of Zeiraphera diniana had the same susceptibility to homologous GV infection (Benz, 1962). Similarly, when the NPV of Phryganidia californica and the GV of Pieris rapae were tested in two populations of the homologous host of each, the LD50 varied by less than 2 fold (Martignoni and Schmid, 1961). When Ripa (1978) assayed Pieris brassicae GV in four West European populations of P. brassicae he found a ten fold variation in LD50. A slightly larger difference was shown in GV susceptibility of sixteen field populations of Phthorimaea operculella which varied 15 fold (Briese and Mende, 1981). Large differences are sometimes found when populations extensively reared in the laboratory are compared with those recently acquired from the field. Klein and Podoler (1978) showed that two field populations of Spodoptera littoralis were about 15 fold more susceptible to NPV infection than a laboratory culture of this species. Similarly Briese et al. (1980) showed that two field populations of Epiphyas postvittana were 50 to 160 fold more susceptible than a laboratory population. The authors attribute this large difference to endemic NPV infection in the stock population which, over several generations, selected for more resistant individuals. David (1978) reported than an increase in resistance of Pieris brassicae to GV infection occurred over two years of laboratory rearing a population. However, Payne et al. (1981) showed that after rearing this species in the laboratory for 15-20 years it showed similar GV susceptibility to that of a field population. Watanabe (1965) found a very large difference in the

susceptibility of two Bombyx mori strains to CPV infection. Here the difference in LD50 was over 300 fold, but this can be ascribed to the fact that these populations were not natural but extensively reared by man with considerable selection pressures.

In summary, the geographical variation in the infectivity of viruses and the susceptibility of their natural host populations is not great. Where populations of insects have been extensively reared in the laboratory there is a risk that they will be less susceptible to infection than their wild counterparts, particularly if virus contamination of the stock insects is a problem.

There seem to be several complexes of virus variants and related hosts, particularly among the Noctuidae, and here the viruses often have similar infectivity regardless of the original species of isolation.

b) Time course studies

Most calculations in the literature of the median lethal time for virus infection have been inaccurate due to two main sources of error. Where lethal times are estimated from an assay where there is continuous feeding on the virus contaminated diet, higher doses will apparently give shorter lethal times, but this is really a reflection of the time taken to acquire a lethal dose. This point is illustrated and discussed by Payne (1981a)). Even where acquisition time is limited, this effect may be important. Boucias and Nordin (1979) assayed a CPV in Hyphantria cunea larvae using a 48 hour feeding period and therefore incorporated a difference of up to two days in lethal time between high and low doses.

More important has been a common misconception of the method for calculating median lethal time by regular checking of assays set up to

estimate the dosage-mortality response. Theoretically, this data cannot be used to calculate the LT50 using probit analysis as the mortality after each time interval is dependent on that at the previous time. However, in these assays a plot of the logarithm of time against probit mortality usually gives such a good fit when drawn by hand that this is the most appropriate method (Litchfield, 1949). However, data used to measure the dosage-mortality response usually results in less than 100% mortality at most doses. Several workers have applied the truncated results method of Litchfield (1949) to deal with these results. They have failed to realise that 'truncated' in his sense means that a 100% response will occur but has not yet done so. With dosage-mortality data at low and intermediate doses the data set is complete with no further response expected.

Assuming that the dosage-response distribution of a population exposed to infection is independent of the distribution of time-response, then it is reasonable to consider that the maximum mortality achieved at a particular dose covers 100% of the time-response distribution. Thus the correct method is to multiply the dosage-response % mortality by a factor giving 100% maximum mortality. The fact that the time mortality curve is sigmoid where maximum mortality is low, and the curve can be made linear by log/probit transformation, confirms the independence of the two response distributions. Using the incorrect method, the time taken to achieve 50% mortality at the LD50 dose is the LT50. In fact this is an overestimate of the true LT50 which should be the time taken to reach 50% of the total mortality, or 25%. The result of this misinterpretation is that many authors have found that LT50 is dose-dependent whereas this result is an artifact of the calculation method. Ignoffo (1964) calculated the LT50 for Trichoplusia ni larvae infected with T. ni NPV as 4.5 days at a dose of 1500 polyhedra/ml and 8 days at 350 polyhedra/ml. Recalculation from his data shows that the values should be 4.2 and 4.9

days, respectively. The slight LT50/dose relationship remaining is particularly obvious at extremely high and low doses and is probably due solely to the continuous feeding method used. Boucias and Nordin (1977(a); 1979) also found dose-related LT50 using the incorrect method. Magnoler (1974) suggested that LT50 was dose-related when he examined the NPV infection of third instar Lymantria dispar larvae. In this case recalculation gives an LT50 of 11-12 days at all doses except at 100 fold and 1000 fold greater than the LD50 when the LT50 is reduced to 10 and 8 days respectively. While it might be expected that low doses would give similar LT50's as a result of the massive multiplication potential of the virus, presumably extremely high doses could slightly reduce the time for infection of all susceptible tissues. Alternatively, such high doses might disrupt the larval physiology so that resistance is lowered and viral development is faster.

Payne et al. (1981) also used the wrong method for LT50 calculation, but by calculating LT50 from the LD90 dose only, the errors were negligible. The LT50's for Pieris rapae and Pieris brassicae first instar larvae with P. brassicae GV were similar at 5.7 and 6.2 days respectively at 20°C. Fourth instar larvae took longer to die with LT50's of 7.4 and 8.9 days respectively. Evans (1981) found that Mamestra brassicae final instar larvae took longer to die than earlier instars when infected with M. brassicae NPV. The LT50 was about 9.5 days for fifth instars and 6.5 days for instars one to four at 22°C.

Temperature differences can make comparison of results difficult as species are usually assayed at their optimum rearing temperature. Studies have shown that while the LD50 is independent of assay temperature, the LT50 is inversely proportional to temperature (Ignoffo, 1966;

Boucias et al., 1980). Thus reanalysis of Charpentier's data (1980) gives an LT50 for Agrotis segetum GV in first instar larvae as 10 days at 17°C and 5 days at 27°C.

There are some systematic differences in LT50 between different types of virus. In general CPV infections have a long LT50. Oryia (Hemerocampa) pseudotsugata CPV infection in fourth instar larvae had an LT50 of 15-20 days (Martignoni et al., 1969). This is similar to the time found by Boucias and Nordin (1979) for a CPV infection of Hyphantria cunea larvae. Baculovirus infections usually have shorter LT50's as indicated above. Among some of the noctuid infections the homologous GV can take longer to kill than the NPV. This is the case with Trichoplusia ni where the LT50 was 7 days for the NPV and 12 days for the GV at the LD50 dose of each (Paschke et al., 1968). A similar difference was found in Heliothis armigera, with values of 6-7 days and 22 days respectively (Whitlock, 1974). The relationship between LT50 and inclusion type may extend to the type of virus particle envelopment. Harper (1976) showed that plusine sNPV's generally took longer to kill their hosts than mNPV's.

It should be noted however that some GV infections have short lethal times, for example F. brassicae GV (Payne et al. 1981) and Cydia pomonella GV (Payne, 1981(a)). It is interesting that these are highly infectious viruses with low LD50's, and that no homologous NPV has been observed in these species.

c) Resistance to Viral Infection

Evans (1961(a)) points out that very little is known of the mechanism governing resistance and susceptibility to virus infection in insects. While insects do not have a true immune system (Tinsley, 1975), the gut

in particular may act as a site mediating infectivity (Section 4(b)(c)). It is noted above that susceptibility to infection decreases with age, a phenomenon often called 'maturation resistance'. Evans (1981(a)) found that the LD50 of M. brassicae NFV for M. brassicae larvae increased at a faster rate in old larvae than expected from the linear LD50/weight relationship found with younger larvae. He argues that these old larvae are showing 'true' resistance. In fact there is no evidence that the link between weight and LD50 is other than circumstantial. It does not exist in all species and where the relationship breaks down it merely confirms that we do not know the mechanism of maturation resistance.

When differences are found in resistance to infection between larval populations, they appear to be genetically controlled. Briese et al. (1980) produced hybrids from Epiphyas postvittana populations which differed in susceptibility to E. postvittana NFV by 100 fold. The hybrids should intermediate susceptibility. The silkworm strains Saizo and Okusa exhibit a 2.5 log difference in susceptibility to Bombyx mori CPV. Here cross-breeding suggests that a dominant major gene may control the effect (Watanabe, 1965). In this species the resistance mechanism is general in effect, as a significant relationship exists between interstrain susceptibility to NFV and CPV (Watanabe, 1966(b)).

By exerting a selection pressure of greater than 60% CPV mortality per generation over five generations of B. mori, Watanabe (1967) produced a population with 16 fold lower susceptibility to the disease. This confirms the genetic control and population lability of resistance. When Whitlock (1977) attempted to produce NFV and GV-resistant populations of Heliothis armigera by similar selection he failed. However, in this

case the selection pressure was not constant and averaged only 54% with NPV and 64% with GV. A close examination of the results shows that the level of mortality oscillated from generation to generation, suggesting that selection could have occurred in one generation producing more resistant larvae. These suffered low mortality at the same virus dose and due to this lowering of selection pressure the susceptibility increased in the next generation. This interpretation is supported by the larger oscillations with GV caused by the greater mean selection pressure. It is possible that a continuous selection pressure rather than continuous dose would have produced a resistant population.

In the natural environment virus selection pressures will often be too low to cause resistance selection (section 7(a)), except in the epizootic situation. The high susceptibilities commonly found in natural populations may be a result of this low selection pressure, a point argued by Boucias and Nordin (1979) to explain the high infectivity of Hyphantria cunea CPV in its host. Payne et al. (1981) note that a population of Pieris brassicae reared in the laboratory for 15-20 years had the same susceptibility to GV infection as a field population. This suggests that this natural population is not under significant viral selection pressure.

(7) Virus Ecology

a) Natural incidence

The incidence of a virus disease can be separated into two components: the number of populations infected and the infection level within populations. Few studies have attempted to do more than estimate the level of infection within a population. However Harcourt (1966) did a key factor analysis on all causes of mortality in Pieris rapae populations

in Canada. He found that the GV was the key factor for generation survival. This virus was also present at high levels in P. rapae populations in Australia (Hamilton, 1979).

The highest incidence levels are found during epizootics. These are particularly common in forest pests where the insect population often oscillates over a few years. Virus infections may be a major cause of collapse of these populations, perhaps due to changes in population resistance as well as density. An example of this phenomenon is the natural collapse of Orgyia pseudotsugata populations due to NPV infection (Mason and Thompson, 1971). A better estimate of the importance of virus diseases can be obtained by studying insect populations at lower, more stable levels. Moore et al. (1973) studied the incidence of NPV, GV and EPV infections in the New Zealand pasture pest Wiseana spp. The NPV infections were most widespread being found in 37% of populations compared with 16% for GV and very few for EPV. NPV was also found at the highest incidence within populations. Most insects at the infected sites showed NPV infection while fewer than 50% had GV or EPV infections. Sherlock (1977, 1978) found rather low levels of virus infection in Agrotis segetum larvae sampled at 41 sites in England. While 25% of sites had some NPV infection the overall incidence of this virus and A. segetum GV was 2%. The problem with such samples is that larvae dying at a very early stage will be poorly represented in the non-random sample. These individuals may represent a large part of the virus mortality and the real incidence will then be much greater than the estimate.

Due to the wide variations in virus incidence which have been reported in the literature, it is not possible to generalise about the importance of these infections as selective agents on insect populations.

McKinley (1979) noted that although Spodoptera NPV's are common in the field he found no evidence of local differences in population resistance or for the development of local virus strains.

b) Transmission and survival

Although vertical transmission of virus can occur by transovum transmission under artificial conditions (Evans and Harrap, 1982; Payne, 1981(b)), it is not clear how important this mechanism is for the natural survival and spread of insect viruses. Infection by most occluded viruses usually results either in host death or, with CPV infections particularly, reduced adult breeding potential (Payne, 1981(b)). This would suggest that direct vertical transmission of virus is uncommon. Etzel and Falcon (1976) for example, failed to find GV transmission from sublethally infected codling moth larvae to their offspring. The main route for virus transmission is probably horizontal, by healthy insects feeding on contaminated material. It is also possible for transmission by vectors to occur. Hyposoter exiguae is an ichneumonid parasite of Trichoplusia ni, and females can transmit T. ni NPV during the oviposition process (Beegle and Oatman, 1975). Reardon and Podgwaite (1976) found a correlation between the incidence of NPV infection in Lymantria dispar populations and parasitoid levels, although there was no evidence of causality.

Where the major transmission route is via contaminated foliage, virus survival outside the host will be important. In temperate climates virus survival over several months may be required in order to infect the next generation. Baculoviruses are very susceptible to inactivation by the ultraviolet component of sunlight. Most ultra-violet (u.v.) radiation penetrating the ozone layer is near u.v. of wavelength 300-380 nm which can rapidly inactivate NPV (Witt and Stairs, 1975). Purified

Pieris brassicae GV on cabbage leaves was completely inactivated by one day of strong sunlight (David et al., 1968). Similarly, purified Trichoplusia ni NPV and P. rapae GV were largely inactivated within ten days on cabbage (Jaques, 1972). Ignoffo et al. (1974(b)) found that Heliothis sp. NPV had a half-life of two to three days on soybean foliage. However, where impure virus is used it persists longer and trees, as opposed to agricultural crops, retain their inoculum for some time (Evans and Harrap, 1982).

Some attempts have been made to artificially select u.v. resistant virus strains. Brassel and Benz (1979) produced Cydia (Laspeyresia) pomonella GV which was twice as persistent in the field, using 315 nm u.v. selection over six virus passages. This might suggest that in the field u.v. selection is not strong. However, when Witt and Hink (1979) selected Autographa californica NPV which was more resistant to near u.v., the infectivity to T. ni larvae was 50 fold less than that of wild virus. In this case there may be a balance between selection pressures for u.v. resistance and infectivity in natural populations. Survival of virus may occur mainly at shielded sites such as in semi-decomposed larval corpses or bird droppings (Entwistle and Adams, 1977) or in the soil and on tree bark (Podgwaite et al., 1979; Jacques, 1974; Jacques and Harcourt, 1971).

It is generally held that the I.B. aids virus persistence (Evans and Harrap, 1982). This is supported by work showing that non-occluded viruses such as Oryctes baculovirus or Panonychus ulmi virus (baculovirus-like) are rapidly inactivated under conditions similar to those in which these viruses naturally occur (Zelazny, 1972, Putman, 1970).

A range of studies on persistence of occluded baculoviruses suggest that these can survive for longer periods under natural conditions (Anderson and May, 1981). It is interesting to note that the Oryctes rhinoceros virus exhibits an extensive gut infection in adults as well as a lethal disease in larvae (Gorick, 1980). These life stages can infect each other at the breeding sites and so there is no need for prolonged virus survival away from the host. The lack of an I.B. in this case may indirectly support the protection hypothesis.

The reproductive potential of occluded viruses must exceed their degradation rate outside the host in order for the virus to survive. NPV infections of Lepidoptera can produce up to 1.5×10^{10} polyhedra per larva, while those of Hymenoptera up to 2×10^8 (Evans and Harrap, 1982). The amount produced is related directly to larval weight, for example the NPV disease of Operophtera brumata produces 2×10^7 polyhedra per mg for all instars (Wigley, 1976).

Larval death and tissue breakdown, seen particularly in baculovirus infections, must be considered advantageous to these viruses which are usually transmitted orally. These processes result in rapid virus dispersal for further infection of the population. Virus spreading between populations may be performed by predators which usually do not dissolve or destroy inclusion bodies as they pass through the gut (Entwistle et al., 1977; Crawford and Kalkmakoff, 1978; Capinera and Barbosa, 1975).

The massive virus inoculum produced by population infection is gradually lost during the interlarval period. The level of Gilpinia hercyniae NPV falls by around 500,000 fold in the overwintering period (Evans and Harrap, 1982). Successful multiplication in the next

generation will depend on optimising infectivity to neonate larvae as soon as they appear. This will cause an initial inoculum increase, allowing widespread infection of older larvae and consequent massive increases in inoculum.

CHAPTER 2 MATERIALS AND METHODS

<u>CONTENTS</u>	<u>PAGE</u>
(1) DECONTAMINATION PROCEDURES	73
(2) THE INSECTS	74
a) SPECIES STUDIED AND ORIGIN OF STOCKS	74
b) MAINTENANCE OF INSECT STOCKS	74
c) DIET COMPOSITION	77
(3) THE VIRUSES	78
(4) PRODUCTION AND PURIFICATION OF VIRUSES	78
a) OCCLUDED VIRUS PREPARATIONS	78
b) SEPARATION AND PURIFICATION OF BACULOVIRUS VIRUS PARTICLES, GRANULIN AND POLYHEDRIN	82
(5) LIGHT MICROSCOPY	83
a) EQUIPMENT	83
b) COUNTING INCLUSION BODIES	83
c) MEASURING POLYHEDRAL DIAMETERS	84
d) EXAMINATION OF LARVAL SMEARS	84
(6) ELECTRON MICROSCOPY	85
a) SPECIMEN PREPARATION FOR THIN SECTIONING	85
b) SECTIONING AND EXAMINATION OF THIN SECTIONS	86
c) NEGATIVE STAINED PREPARATIONS	86
d) SCANNING ELECTRON MICROSCOPY	86
(7) BIOCHEMICAL ANALYSES	87
a) SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF POLYPEPTIDES	87
b) NUCLEIC ACID PURIFICATION	88
c) RESTRICTION ENDONUCLEASE DIGESTION OF BACULOVIRUS DNA	89

(7) a)	AGAROSE GEL ELECTROPHORESIS OF NUCLEIC ACIDS	90
e)	POLYACRYLAMIDE GEL ELECTROPHORESIS OF NUCLEIC ACIDS	91
(8)	SEROLOGICAL ANALYSES	92
a)	PREPARATION OF ANTISERA	92
b)	IMMUNODIFFUSION	93
c)	PREPARATION OF γ -GLOBULIN	93
d)	ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)	93
(9)	INFECTIVITY ASSAYS	94
a)	DOSING METHOD	94
b)	ESTIMATION OF DOSE VARIABILITY ON LEAF DISCS USING ^{32}P -LABELLED VIRUS	96
(10)	PRODUCTION AND COUNTING OF ^{32}P - AND ^3H -LABELLED VIRUS	97

CHAPTER 2

MATERIALS AND METHODS

(1) Decontamination Procedures

For precise studies of the infectivity and host range of insect viruses, it is necessary to observe strict procedures to prevent virus contamination of stock insects or experimental insects.

Virus inactivation on used glassware was carried out by soaking overnight in a 5% solution of 'Chlorox' (Imperial Chemical Industries Ltd.) followed by washing and drying at 98°C. Contaminated plasticware was discarded. Reusable plasticware was soaked overnight in 5% 'Chlorox' before washing. Centrifuge tubes were decontaminated by soaking in 2% formaldehyde overnight and rotor buckets were filled with this solution after use. All bench surfaces were swabbed with a 5% solution of 'Domestos' (Lever Industrial Ltd.) before and after experiments.

Metal instruments were placed in an oven at 95°C overnight after use. Where infected insects were handled individually, the instruments used were heated, plunged into alcohol and flamed off between each handling. Paintbrushes and similar heat sensitive instruments were decontaminated in a saturated formaldehyde atmosphere overnight.

Virus-contaminated disposable solid materials were incinerated and liquids were mixed with formaldehyde at a final concentration of at least 2% before disposal.

(2) The Insects

a) Species studied and origin of stocks

Six noctuids from three subfamilies, and one pierid were used. These are listed in Table 1, together with details of their biology, food plants and pest status.

A. segetum stocks were originally obtained from Dr. F. Sherlock (Rothamsted Experimental Station, Harpenden, U.K.). These were supplemented with adults caught in a light trap at the Glasshouse Crops Research Institute (G.C.R.I.). Initial stocks of M. brassicae were supplied by Dr. H. Evans (Institute of Virology, Oxford, U.K.), but these were entirely replaced by locally caught insects with which most work was done. All other noctuid species were obtained locally. The light trap used was a Robinson trap with U.V. lamp (Reid, 1979). P. rapae larvae were obtained from a continuous culture of this species reared at the G.C.R.I. for several years and originating from a local population (Payne et al., 1981).

b) Maintenance of insect stocks

Continuous rearing of the noctuids was carried out throughout the study, with the exception of N. promuba. The adults of this species aestivate if the day length exceeds 15 hours and the preoviposition period can be very long (Novak and Spitzer, 1975).

All work with N. promuba was therefore done using the first generation obtained from field collected adults, reared with 14 hour daylight periods.

The other noctuids were maintained at 18 hour daylight in a room where the relative humidity was 50% and the temperature 20°C.

TABLE 1 THE LEPIDOPTEROUS SPECIES STUDIED

FAMILY	SUB-FAMILY	SPECIES	COMMON NAME	BIOLOGICAL NOTES	FOOD PLANTS, DISTRIBUTION AND PEST STUDIES
NOCTUIDAE	NOCTUINAE	<u>Agrotis segetum</u> (Schiff.)	Turnip Moth	5-7 Instars. One generation (or partial 2nd) in Brit. Larval overwintering.	Cutworms. Feed on foliage during early instars, then subterranean from 3rd instar onwards. Wide range of host plants including potatoes, swedes, turnips, oats, beans, carrots, chrysanthemums. Found in Europe, Asia, N. Africa. <u>A. segetum</u> is possibly the most serious noctuid pest.
		<u>A. exclamatoris</u> (L.)	Heart & Dart Moth	6-7 Instars. Similar to above.	
		<u>Noctua promba</u> (L.)	Large Yellow Underwing	7 Instars. Similar to above.	
	HADENINAE	<u>Mamestra brassicae</u> (L.)	Cabbage Moth	6 Instars. One generation (or partial 2nd). Pupal overwintering.	Omnivorous. Cabbage, turnips, lettuce, rape, peas etc. Common in Europe and Asia. Serious pest in E. Europe & Russia. Occas. pest of glasshouse carnations and chrysanthemums.
		<u>Laconobia oleracea</u> (L.)	Tomato Moth	5-7 Instars. One generation. Pupal overwintering.	Feeds on range of crucifers, mainly cabbage, swedes. Found in Europe, Asia & N. Africa. Occas. pest of tomatoes under glass.
	PLUSIINAE	<u>Plusia gamma</u> (L.)	Silver Y	5-6 Instars. Immigrant. Not known to overwinter in Brit.	Variety of vegs. including cabbage, peas, beans, potatoes, onions, turnips. Found in Europe, Asia & N. Africa. Pest of chrysanthemums under glass.
PIER.		<u>Pieris rapae</u> (L.)	Small White Butterfly	5 Instars. Two generations. Pupal overwintering.	Wide ranging pest of brassicas, turnips, swedes, mustard. World wide distribution includes Japan, N. America, New Zealand, as well as Europe. Probably the most serious butterfly pest.

KEY: PIER. = PIERIDAE; PLUSII. = PLUSIINAE.

References: Smith (1949); Cayrol (1972); Hussey et al. (1969)
Gomaa (1978); Bretherton, et al., (1979)

Occasionally, higher or lower temperatures were used with particular batches in order to regulate the developmental period. Adults were sexed by examination of antennae (Agrotis spp.), wing coloration (N. pronuba) or external genitalia. Most species were allowed to mate in glass lamp-jars in which strips of tissue paper were fastened as oviposition sites. N. pronuba adults were placed in a large brown paper bag for this purpose and some batches of P. gamma adults were found to oviposit more efficiently in bags. The adults' food source was a solution of 5% sucrose placed in a small pot with tissue paper wick.

Eggs were removed still attached to the pieces of paper on which they had been laid. They were fumigated by exposure to an atmosphere of 12.5 mg formaldehyde dm⁻³ for 6 hours (Bathon and Groner, 1977). The eggs were then placed in plastic tubs (Borden U.K. Ltd.) together with small lumps of semi-synthetic agar based diet (Section 2(c)). The lid of each tub was slit for ventilation and a piece of tissue paper was placed under it to absorb moisture.

Larvae intended for stocks were transferred individually to small plastic pots (Mardon Illingworth Ltd.) when they reached third instar. Here the lids were slit and excess moisture absorbed by filter paper. Separate rearing at this stage prevents the cannibalism which is often found, particularly in the cutworms. Pupation occurred in these pots and the pupae were removed and placed on filter paper in a plastic tub. P. gamma pupates in a cocoon which was removed to allow easy emergence. This rearing method was found to be successful with all the noctuid species tried. It is more convenient than most other systems which have been used, for example with M. brassicae (David and Gardiner, 1966) and A. segetum (Sherlock, 1979). There was no problem with death of A. segetum neonates caused by high humidity (Zethner, 1980(a)). Nor was

it necessary to use a pupation medium of peat or vermiculite (David and Gardiner, 1966; Sherlock, 1979).

No overt signs of virus infection in the insect stocks were found at any time. This contrasts with reports by other workers, for example Martignoni et al. (1973) rearing P. gamma at the G.C.R.I. The use of disposable materials, particularly when rearing young insects, may be an important factor in preventing outbreaks of virus in insect stocks. Very occasionally vestigial winged A. segetum adults were found. This did not appear to be related to sublethal GV infection as was suggested by Charpentier (1980).

Rearing of P. rapae was carried out using a more complex system described by Payne et al. (1981).

c) Diet composition

A simple semi-synthetic diet was used for all the noctuid species. It has been described by Guennelon et al. (1981). As well as being applicable to a variety of species, this diet is cheaper to produce than many others (Payne, pers. comm.) and is less prone to fungal and bacterial contamination despite the absence of antibiotics.

Composition of Noctuid Diet

Water	1000.0 g
Agar	26.6 g
Maize meal	190.0 g
Wheatgerm	50.0 g
Yeast powder	46.0 g
Ascorbic acid	7.0 g
Benzoic acid	3.0 g
Methyl para-hydroxy benzoate	2.3 g

The diet was made by dissolving the agar in 600 ml H₂O, cooling to 70°C and adding to the other ingredients, premixed in 400 ml H₂O.

P. rapae was reared on an agar based diet described by David and Gardiner (1965). This contains dried powdered cabbage to stimulate feeding. The main sources of protein and carbohydrate are casein, wheatgerm and sucrose. A complex mixture of salts and vitamins are included.

Both diet types were prepared in an area free from virus contamination and it was not necessary to add formalin. The same diet batch could thus be used both for stock rearing and experimental purposes. Storage was successful at -20°C or 4°C.

(3) The Viruses

Table 2 lists the main virus isolates which were studied, together with the country of original isolation (where known) and supplier of the isolate. In this study the convention of naming the virus after the host from which it was originally isolated will be followed (but see Chapter 8). Table 2, together with characterisation data presented in Chapter 3, clarifies the origin and characteristics of the particular variants which were studied.

The virus isolates were passaged at least once at the G.C.R.I. in the homologous host (i.e. that from which the virus was originally isolated) before experimental use.

(4) Production and Purification of Viruses

a) Occluded virus preparations

For production of baculoviruses or CPV's, late instar larvae of the appropriate species were individually dosed with a suspension of I.B.'s

TABLE 2 ORIGIN OF MAIN VIRUS ISOLATES STUDIED

VIRUS	COUNTRY IN WHICH ORIGINALLY ISOLATED	REFERENCE TO ORIGINAL ISOLATION	INOCULUM SUPPLIER	NOTES
<u>A. segetum</u> mNPV	England	Sherlock (1977)	Dr. P. L. Sherlock. Rothamsted Exp. Station, Harpenden, U.K.	—
	France	Burgerjon <u>et al.</u> (1975)	Dr. A. Burgerjon, Inst. Biological Control, La Minière, France.	—
	Poland	Lipa <u>et al.</u> (1971)	Dr. J. J. Lipa. Inst. Plant Protection, Poznan, Poland.	—
<u>M. brassicae</u> mNPV	Germany	Gröner (1976)	Mr. D. A. Brown. Institute of Virology, Oxford, U.K.	Passaged at Oxford U.K.
<u>P. gamma</u> sNPV	Unknown	None	Dr. H. F. Evans. Institute of Virology, Oxford, U.K.	2 isolates obtained from Inst. of Virology collection. Origin unclear. Fully described in Ch. 3.
<u>A. segetum</u> GV	Austria	Zethner (1980(b))	Dr. O. Zethner. Royal Vet. & Agric. University, Copenhagen, Denmark.	Passaged at Darmstadt F.R.G.
<u>P. brassicae</u> GV	England	Smith and Rivers (1956)	Dr. C. C. Payne. G.C.R.I.	Passaged at Oxford U.K.
<u>P. gamma</u> CPV	England	Martignoni <u>et al.</u> (1973)	Dr. C. C. Payne G.C.R.I.	—

KEY: mNPV = nuclear polyhedrosis virus with some multiply enveloped nucleocapsids; sNPV = nuclear polyhedrosis virus with all singly enveloped nucleocapsids; GV = gramlosis virus; CPV = cytoplasmic polyhedrosis virus.

in water. Several hundred larvae were used to produce the main virus preparations used in this study, but one hundred larvae or less were sufficient for production of virus progeny for identification in cross-transmission experiments.

Baculovirus-infected larvae were collected at death, placed in 0.1% sodium dodecyl sulphate (S.D.S.) and stored frozen at -20°C before virus extraction. Dead larvae were used in preference to moribund ones from which less infective 'immature' I.B.'s may be obtained (Ignoffo and Shapiro, 1978). CPV infections often do not lead to death in older larvae (Payne, 1981(b)) and in CPV-infected larvae the guts were removed approximately two weeks after the initial infection.

I.B.'s were released from larval material by treating for one minute in a 'Colworth Stomacher 80' (A. J. Seward, London), or in the case of some CPV preparations by maceration in a ground-glass tissue grinder. The resulting suspension was filtered through two layers of muslin and washed with 0.1% S.D.S. Further purification was carried out by cycles of differential, rate-zonal and isopycnic centrifugation. Low speed centrifugation was done on a Sorvall RC-2 with SS 34 (angle-head) and HB4 (swing-out) rotors, or a Sorvall RC 5B with SS 34 or HS 4 (swing-out) rotors. For ultracentrifugation a M.S.E. PrepSpin 50 was used with 6 x 16.5 ml or 3 x 70 ml aluminium swing-out rotors. Glycerol gradients were formed using an M.S.E. gradient maker.

The following schedules were used for purification of GV or polyhedral I.B.'s: ($K = 10^3$ r.p.m.)

<u>NPV/CPV</u>	<u>GV</u>
(1) Removal of large contaminants:	
1 K briefly	2 K 5 minutes
repeat with pellet; combine supernatants, discard pellet	

<u>NPV/CPV</u>	<u>GV</u>
(2) Removal of lipid and small contaminants:	
5 K 10 minutes	10 K 30 minutes
retain pellet, discard supernatant	
(3) Rate-zonal gradients:	
30-90% glycerol (v/v) in	30-80% glycerol (v/v) in
0.1% S.D.S.	0.1% S.D.S.
3.5 K 8 minutes	10 K 40 minutes
retain virus band and pellet it	
(4) Isopycnic gradients:	
45%-60% sucrose (w/w) layers in 0.1% S.D.S.	
20 K 1 hour	
Retain I.B. band and recover by low speed centrifugation	
Wash twice in distilled water	

The rate zonal stage was often found unsatisfactory with the polyhedral viruses, partly because of variation in I.B. size within any isolate. Unless very low loads of polyhedra were used, poor separation was achieved, with I.B.'s aggregating and pelleting. Omitting this stage did not appear to reduce the purity of the final preparation as judged by phase contrast microscopy. This is probably due to the ease with which contaminants are removed from polyhedra by differential centrifugation. GV capsules were successfully purified on rate-zonal gradients due to their size homogeneity.

I.B. preparations were stored as suspensions in water at -20°C for long-term storage, or at 4°C for short periods. Previous studies have

shown that occluded viruses can be stored under these conditions for several years with little or no activity loss (Dulmage and Burgerjon, 1977; Lewis and Rollinson, 1978).

The protein concentrations of I.B., virus particle and granulin or polyhedrin preparations were estimated by the method of Lowry et al. (1951).

b) Separation and purification of baculovirus virus particles, granulin, and polyhedrin

I.B. suspensions at between 5 and 20 mg/ml were dissolved by addition of an equal volume of 0.1 M Na_2CO_3 for 20 minutes at room temperature, or for 30 minutes at 4°C where endogenous proteolytic activity was to be reduced. Dissolution was stopped by lowering the pH with an equal volume of 0.05 M tris/Hcl buffer at pH 7.8. Any undissolved I.B.'s were removed by low speed centrifugation. The supernatant was then placed on 30-65% (w/w) sucrose gradients buffered with 0.05 M tris/Hcl pH 7.8. Centrifugation to equilibrium was achieved at 25,000 r.p.m. for 3 hours. Polyhedrin or granulin was removed from the top of the gradient and dialysed against distilled water or buffer. Virus particle bands were pooled and pelleted twice in distilled water. Both components were stored at -20°C.

Virus particle degradation is reduced by using buffered gradients and lowering the pH after a limited dissolution period (McCarthy and Liu, 1976). Good yields of virus particles were obtained, approximately 2.5% of total NPV I.B. protein being recovered as virus particle protein.

(5) Light Microscopy

a) Equipment

Zeiss standard R.A. microscopes with 2x Optovar magnification changer were used with a planapo 100x oil immersion objective for critical observation of dried smears of viral I.B.'s. A Zeiss camera body was used for photography, and exposure was determined with a photocell and Zeiss four stage photometer.

b) Counting inclusion bodies

Various methods for counting purified I.B. preparations were tested. Haemocytometer counting with 0.01 mm and 0.02 mm depth Thoma ruled slides (Gelman-Hawksely Ltd., Lancing, Sussex) and dried smear counting (Wigley, 1980) gave comparable results with polyhedra. Dried smear counting was found to be unsuitable for GV capsules, however, as their small size prevented them from being clearly distinguished. Therefore haemocytometer counting was adopted as a standard method for all I.B. types. The 0.02 mm depth chamber was used as it gave less variable results than the 0.01 mm chamber. This is probably because the major source of error is variation in chamber depth between counts (Burgess and Thomson, 1971) which is proportionately less important as depth increases. 0.1 mm depth chambers were too deep to allow counting of I.B.'s without continuous refocusing. The 0.02 mm depth chamber was used with phase contrast optics in the case of NPV I.B. counting, or dark field optics for GV's. The stock suspension was diluted so that about 5-10 I.B.'s lay in each small square ruled on the haemocytometer. About 2 μ l of suspension were put on the slide which was gently moistened by breathing to improve coverslip adhesion (Burgess and Thomson, 1971).

I.B.'s were counted in squares on a diagonal across the slide until at least 400 had been counted. The number of I.B.'s in the original suspension was then calculated. For each suspension two replicate dilutions were made and each counted twice. If there was a large variation in the counts, further replicate counts were done. The final result was taken on the mean of these counts. The slide and coverslip were washed in alcohol between each count.

Using this method the standard error (S.E.) of the count is the square root of the number of I.B.'s counted (Burgess and Thomson, 1971). However, differences between replicates are often larger than this would suggest as a result of systematic errors.

c) Measuring polyhedral diameters

Light microscopy can be used to rapidly measure large numbers of polyhedra. Bright field illumination is not satisfactory as it produces a diffraction pattern at the edge of objects and judging the position of the edges is impossible (Ross, 1957). Using phase contrast illumination of dried smears this problem is eliminated since the polyhedra then have sharp outlines (see also Section 5(d)). Polyhedra were measured using an image-splitting eyepiece (Vickers-A.E.I., England). This was calibrated by reference to a stage micrometer with 10 μ m ruling.

d) Examination of larval smears

A variety of methods have been devised which are useful for diagnosis of larval infections by light microscopy, using either wet mounts or stained larval smears (Payne and Kelly, 1981). Most of these methods are too time consuming for rapid diagnosis of large numbers of infections. In this study a simple but effective method was used which was suitable for diagnosis of death or infection by occluded viruses

or bacteria. Phase contrast microscopy of dried larval smears was used, with an oil immersion 100x objective. Baculovirus I.B.'s appear dark grey by this method, GV capsules being clearly distinguishable from rickettsiae and other similarly sized bodies. CPV I.B.'s are lighter in appearance with a sharp outline and are distinct from NPV polyhedra.

(6) Electron Microscopy

a) Specimen preparation for thin sectioning

Examination of thin sections was carried out both on purified I.B. preparations (Chapter 4) and on neonate larvae which had been fed virus (Chapter 7).

I.B. specimens were prepared in polythene embedding capsules (TAAB laboratories), the sample (0.2-1.0 mg) being pelleted in a bench centrifuge at each fluid change. For optimum penetration of fixatives etc. into neonate larvae it was necessary to remove the head and the abdomen distal to the area to be examined. The remaining pieces were transferred between fluids using glass tubes with stainless steel gauze filters (Pegler and Atkey, 1978).

The following schedule was used, with 0.2 M phosphate or cacodylate buffer (pH 7.2) as diluent and for washing, unless otherwise stated:

- | | | |
|---------------|-----------------------------|----------------|
| (i) Fixation: | 2% Glutaraldehyde, | 2 hours |
| | wash, | 5 x 20 minutes |
| | 2% aqueous OsO_4 ; | 1½ hours |
| | wash, | 5 x 20 minutes |

(ii) Dehydration: 25%; 50%; 70%; 80%; 90%;

100% ethanol, 30 minutes each

100% ethanol, 2 further washes

(iii) Embedding: Using Spurr's resin (Spurr, 1969)

25% Resin in ethanol 1 day

50% Resin in ethanol 1 day

75% Resin in ethanol 2-3 days

100% Resin in ethanol 2 days

100% Resin in ethanol harden in oven 70°C overnight

I.B. preparations were polymerised in polythene TAAB capsules. Larvae were placed in small flat embedding moulds and orientated before hardening.

b) Sectioning and examination of thin sections

Resin blocks were trimmed with an LKB Pyramitome and sectioned on an LKB Ultramicrotome III using glass knives. Square sections were cut at a thickness of about 75 nm and collected on uncoated copper grids. They were stained with uranyl acetate for 1 hour and lead citrate (Reynolds, 1963) for 15-30 minutes. The electron microscope used was a Jeol JEM 100S operating at 80 kV.

c) Negative stained preparations

This technique was used to examine dissolved I.B.'s and virus particles. The sample was placed on a carbon coated grid and stained with potassium phosphotungstate at neutral pH.

d) Scanning electron microscopy

A Jeol JSM-T20 scanning EM was used to examine I.B. morphology. A drop of purified I.B. suspension was placed on the end of the specimen mount.

This was coated with gold/palladium using an EM-Scope sputter-coater. Photographs were taken at an angle of 60° to the face of the specimen mount.

(7) Biochemical Analyses

a) SDS-polyacrylamide gel electrophoresis of polypeptides

A discontinuous vertical slab gel system was used, with sample, gel and reservoir buffers specified by Laemmli (1970). The equipment used was based on the design of Studier (1973), using glass plates and perspex spacers and combs. The lower gel was 10% acrylamide + 0.3% MBA and had dimensions of 15 x 12.5 x 0.15 cm. A 3% acrylamide + 0.1% MBA gel layer was placed on top. This had a depth of 1 cm with 12 sample wells separated by 0.4 cm wide gel fingers.

Samples, containing 0.1-5 μ g protein per band, were added to 50-200 μ l sample buffer containing 0.002% bromophenol blue and heated to 90°C for 2 minutes. When cool they were applied to the gel which was run at 15 mA until the bromophenol blue marker had completely entered the lower gel. The current was then increased to 30 mA until the dye was within 1 cm of the lower gel edge when the run was stopped. The total run time was around $4\frac{1}{2}$ hours.

Gels were stained in 0.05% Coomassie blue in methanol: acetic acid: water (5:1:5) for 2 hours at 37°C or overnight at room temperature. Destaining used 7.5% acetic acid, 10% ethanol in distilled water.

The following molecular weight standards were used for calculation of virus polypeptide molecular weights. (Crook, 1981(a) unless otherwise stated.)

myosin (200,000); phosphorylase a (97,400) (Fletterick and Madsen, 1980); transferrin (77,000); bovine serum albumin (69,000); ovalbumin (45,000); lactic dehydrogenase (35,000); carboxypeptidase a (29,000); α -chymotrypsinogen (25,700); myoglobin (17,200); cytochrome C (12,400).

b) Nucleic acid purification

Both baculovirus DNA and CPV RNA were extracted directly from purified I.B.'s without further purification of virus particles. Phenol extraction procedures were used in both cases, the main difference being the temperature conditions. Extraction with phenol at 60°C pH5, followed by rapid cooling results in degradation and removal of DNA in the phenol phase which is suitable for RNA purifications (Scherrer, 1969). DNA is purified with phenol at room temperature and neutral pH.

(i) Schedule for DNA purification

Five to 20 mg I.B.'s in 0.5 to 1.0 ml water were dissolved by addition of an equal volume of 0.1 M Na_2CO_3 for 40 minutes at room temperature. Virus particles were disrupted by the addition of SDS to a final concentration of 1% and incubation for 60 minutes at 37°C.

An equal volume of phenol saturated with 0.01 M tris/Hcl buffer (pH 7.6) + 0.001 M EDTA was added to the dissolved I.B. suspension. Gentle shaking for 5 minutes was followed by low speed centrifugation to separate the phases. The lower (phenol) phase was discarded but the interphase was retained with the aqueous layer. Phenol extraction was repeated twice on which occasions the interphase was also discarded. The aqueous phase was then either dialysed extensively against 0.01M tris/Hcl + 0.001 M EDTA to remove traces of phenol and concentrated by dialysis against polyethylene glycol, or the DNA was precipitated with alcohol. In the latter case the aqueous phase was washed in two volumes of ether to remove

phenol, and the DNA precipitated by addition of 2 volumes of ethanol at -20°C . This was left overnight at -20°C and then pelleted by low speed centrifugation, followed by washes in alcohol and ether. The DNA was then resuspended in a small volume of extraction buffer. DNA was stored at a concentration of 100-200 $\mu\text{g/ml}$ (absorbance of 2-4 at 260 nm) at 4°C .

(ii) Schedule for RNA purification

Five to 20 mg of CPV I.B.'s were suspended in 0.01 M sodium acetate pH 5.0, 0.05 M sodium chloride and extracted three times with 90% phenol at 60°C (Payne and Tinsley, 1974). On each extraction the mixture was left for three minutes at 60°C and vortex-mixed four times during this period. It was then placed on ice for a few minutes before separating the phases by low speed centrifugation. The interphase was retained at the first extraction and discarded at the last two extractions. The RNA was precipitated using the method described above for DNA. It was resuspended in a small volume of Laemmli sample buffer (Laemmli, 1970), and stored at -20°C . It was found that RNA samples in this buffer could be successfully used on all the gel systems which are described below.

c) Restriction endonuclease digestion of baculovirus DNA

One to 2 μg of purified baculovirus DNA, or 0.5 μg of bacteriophage λ DNA, was digested in a reaction volume of 15 μl with 5-10 units of EcoRI, Hind III or BamHI restriction enzymes (Miles Laboratories Ltd.) in the appropriate buffer (all pH 7.5):

	<u>EcoRI</u>	<u>Hind III</u>	<u>BamHI</u>
tris/Hcl	100 mM	6 mM	100 mM
NaCl	50 mM	50 mM	—
MgCl ₂	10 mM	6 mM	10 mM

Digestion was continued for 3 hours (EcoRI) or 4 hours (Hind III, BamHI) at 37°C. The reaction was stopped by the addition of 2 µl of 0.1 M EDTA, 20% Ficoll, 0.1% bromophenol blue and heating at 60°C for 10 minutes.

d) Agarose gel electrophoresis of nucleic acids

This was the main electrophoretic system used to separate restriction endonuclease fragments of baculovirus DNAs. It was found that CPV ds RNA genome segments could also be separated using this system which was particularly appropriate for checking possible mixed baculovirus/CPV infections (Appendix 1).

Agarose gels at concentrations 0.7, 1.0 or 1.4% were made up in electrode buffer: 40 mM tris/Hcl; 20 mM sodium acetate; 2 mM EDTA pH 7.8. Dissolved agarose was poured onto a 20 x 20 cm glass plate to a depth of 2.5 mm. Wells 7 x 1.5 mm x 2 mm deep were formed with a perspex mould 2 cm from the end of the plate. Electrode buffer was run in until flush with the upper gel surface. The apparatus was equilibrated by running at 35 V for 30 minutes before adding the samples. Electrophoresis was carried out at 35 V or 40 V for 16-24 hours.

Gels (DNA and RNA) were stained with ethidium bromide at 2 mg/ml in electrode buffer and photographed under short wave u.v. illumination (302 nm), using Ilford FP4 film and a Kodak Wratten No.4 (yellow) filter.

DNA from bacteriophage λ was digested with EcoRI or Hind III restriction enzymes to provide molecular weight standards.

e) Polyacrylamide gel electrophoresis of nucleic acids

Two polyacrylamide gel systems were used for separation of CPV ds RNA genome segments. Baculovirus and λ DNA genome restriction enzyme digest fragments were also tested on these systems (Appendix 1).

(i) 3% acrylamide

Payne and Tinsley (1974) described a 3% continuous gel system for CPV genome segment electrophoresis and this is the main system with which the nucleic acid of these viruses has been analysed since then. When this method was adapted to vertical 15 x 13.5 x 0.15 cm slabs it was found that the gel and electrode buffer described above for agarose gel electrophoresis was more suitable than that described by Payne and Tinsley (1974). Electrophoresis could be completed in a much shorter time (13 hours at 60 V compared with approximately 45 hours) without necessity for renewal of electrode buffer. The results obtained were the same using either buffer system.

The 3% gels were made up as follows:

10% of a stock solution of 30% acrylamide + 0.8% methylene bisacrylamide;
10% 0.4 M tris, 0.2 M sodium acetate, 20 mM EDTA adjusted to pH 7.8 with Hcl;
80% H₂O + 0.02 g ammonium persulphate;
25 μ l TEMED.

The concentration of methylene bisacrylamide is half of that used by Payne and Tinsley (1974), but the same as that used by Laemmli (1970).

(ii) 10% Laemmli discontinuous acrylamide

The Laemmli (1970) polypeptide electrophoresis system has been applied to ds RNA segments from a range of segmented genome viruses, such as Reovirus (Ramig et al., 1977) and Rotavirus (Rodger and Holmes, 1979).

In this study the CPV ds RNA segments were analysed on vertical 15 x 13.5 x 0.15 cm slab gels using the 3% acrylamide upper and 10% acrylamide lower gel concentrations as described by Laemmli (1970). Electrophoresis was carried out at a constant current of 20 mA for 15 hours.

Gels were stained and visualised as described in Section (d).

(8) Serological Analyses

a) Preparation of antisera

Antisera were prepared to virus particles, polyhedra and I.B.'s of A. segetum NPV (English isolate) and M. brassicae NPV. Antiserum to A. segetum GV capsules was provided by Dr. N. E. Crook and had been prepared in the same manner.

Rabbits were immunised by intramuscular injection on days 1, 8 and 15 with 5 mg antigen (polyhedrin and I.B.'s) or 0.5-1 mg (virus particles). These were emulsified with Freund's complete adjuvant on the first injection and Freund's incomplete adjuvant for subsequent injections. Rabbits were exsanguinated 3 to 4 weeks after the final injection, the exact time being dependent on results of gel diffusion tests of 10 ml bleeds at 2, 3 and 4 weeks after the final injection.

Blood was allowed to clot at 37°C for 30 to 45 minutes and then cooled to 4°C for 24 to 36 hours. Serum was removed and centrifuged at 1000 r.p.m. for 5 to 10 minutes. Serum was stored at -20°C without preservatives.

b) Immunodiffusion

Five ml of 0.8% agarose in 0.02 M phosphate plus 0.15 M NaCl, pH 7.4 (PBS) was poured into 5 cm diameter Petri dishes. Wells with a diameter of 6 mm were cut 4 mm apart and 50 μ l of antigen or antiserum added to each well. Antiserum was used undiluted in most cases. Where the antigens were I.B.'s, these were dissolved with 0.1 M Na_2CO_3 plus 1% Nonidet P40 and brought to neutral pH by dilution to 1 mg/ml with PBS.

Gels were left for 24-48 hours at 4°C, washed with PBS and visualised by dark ground illumination, or by staining with Gurr's Brilliant Crocein using the method of Crowle and Cline (1977).

c) Preparation of γ -globulin

γ -globulin was purified from antiserum for use in enzyme-linked immunosorbent assays (ELISA). Antiserum was diluted 10-fold and an equal volume of saturated ammonium sulphate added to precipitate the γ -globulin. After standing for 30 minutes the precipitate was pelleted by low speed centrifugation and dissolved in PBS. After extensive dialysis against PBS the γ -globulin was adjusted to 1 mg/ml (absorbance at 280 nm = 1.38) and stored at -20°C.

d) Enzyme-linked immunosorbent assay (ELISA)

γ -globulin was conjugated with alkaline phosphatase (Sigma type VII) by the method of Avrameas (1969) as described by Clark and Adams (1977). Indirect and double antibody sandwich ELISA were carried out essentially as described by Crook and Payne (1980). For the indirect method plates were coated with dissolved I.B.'s. These were prepared by addition of an equal volume of 0.1 M Na_2CO_3 for 20 minutes followed by dilution to the appropriate concentration in PBS. γ -globulin was used

at a concentration of 5 µg/ml, diluted in PBS plus 0.05% Tween 20 (PBS-Tween). For the double antibody sandwich method the plates were coated with 1 to 3 µg/ml γ -globulin in PBS. The antigens were I.B.'s, dissolved as described above and diluted in PBS-Tween.

p-nitrophenyl phosphate (Boehringer Corporation, London, Ltd.) was the substrate, at 0.1% in 10% diethanolamine-HCl, pH 9.8. After a 90 minute incubation at room temperature, colour intensity was measured at 405 nm using a Beckman model 25 spectrophotometer.

(9) Infectivity Assays

a) Dosing method

A standard method was developed for dosing larvae with virus both in cross-infection tests and dosage-mortality assays. It was found suitable for all the species in this study and it allowed defined doses to be fed to larvae of these species. The method involved spreading virus on a large leaf disc from which small discs were cut for feeding to individual larvae. An experiment using radiolabelled virus was carried out in order to check that the dose on each small disc corresponded to the calculated value and that variation in this dose was small in relation to total dosage-mortality response variation. This experiment is described in Section 9(b).

A cork borer was used to cut 10 mm diameter leaf discs from cabbage leaves (cv. May Express). Flat areas of mature leaves were chosen. The discs were placed upside down on solidified 3% agar to hold them in place and prevent desiccation during virus application. Each of these discs corresponded to one dose/replicate for 25 larvae. For dosage-mortality assays four doses at half $\log_{10}$ intervals were used in

addition to controls for each assay, with two replicates of 25 larvae at each dose. Suitable virus dilutions were made using an aqueous solution of 0.2% agar (Oxoid No.3), 0.3% Teepol as diluent. At this concentration the agar remains fluid at room temperature but has no convection currents during drying. The teepol acts as a wetting agent. Five μ l of diluted virus was spread on a disc with a round-ended glass rod (3 mm diameter), which was first wetted with the suspension to prevent net removal of virus on the rod. When the smear had dried, 25 discs of 0.65 or 0.75 mm diameter were cut and individually placed virus upwards on drops of solidified 3% agar in microtitre plate wells. A sharp stainless steel or brass tube was used for cutting these discs, with a central wire to push out the disc. Discs were prepared in order of increasing virus dose in order to obviate sterilisation of the cutter between doses.

Larvae, within 24 hours of hatching, were placed individually in the microtitre plate wells which were then sealed with 'Cling Film' (Payne Scientific, London). The plates were inverted with all species except Agrotis spp. in order to maximise the feeding rate. After 24 hours, larvae which had not finished feeding (usually because they had suffered handling injury) were discarded. Those which had eaten their discs entirely were reared individually on formalin-free diet in square plastic compartmentalised petri dishes. These were sealed with 'Cling Film'.

During the bioassay larvae were maintained at 20°C with an 18 hour day. Assessment of mortality was carried out daily by smearing all corpses and examining them as described in Section 5(d).

b) Estimation of dose variability on leaf discs using ^{32}P -labelled virus

^{32}P -labelled A. segetum NPV polyhedra were produced as described in section (10). A suspension of approximately 1.8×10^9 I.B./ml had an activity of 2.83×10^6 counts per minute (c.p.m.) per ml as measured by Cerenkov counting (Section 10). A 50 μl aliquot of this suspension was centrifuged at low speed to pellet the I.B.'s which were resuspended in 50 μl of 0.2% agar plus 0.3% teepol. Four portions of 5 μl were taken from this suspension and smeared on four 10 mm cabbage discs from each of which 10 small discs were cut as described in Section 9(a). Each of these was homogenised in a tissue grinder and the amount of radio-labelled virus estimated by Cerenkov counting.

Results: The mean of the counts of 10 small discs from each of the 4 large leaf discs were as follows (after subtraction of background count):

57.2; 58.4; 59.6; 72.0 c.p.m.

The expected count per small disc, based on the virus dose applied, was 57.0 c.p.m. This shows that the smearing method used results in little or no net gain or loss of virus on the small leaf discs, at least in terms of mean dose per disc. The variation in dose per small disc was assessed by combining results from all 40 discs. It was found that the $\log_{10}$ c.p.m. exhibited the frequency distribution shown in Figure 2.1. This distribution was tested by the Kolmogorov-Smirnov non-parametric test (Meddis, 1975) and found not to differ significantly from a normal distribution ($p > 0.1$).

The standard deviation of the distribution was 0.206. The effect of this on the slope of the final probit analysis is given by the following formula adapted from Finney (1952, pp.170-171) : $\tilde{b} = b / \sqrt{1 + S_2^2/S_1^2}$

where, $\bar{b}$ is the final probit slope,

b is the probit slope if dose variation were zero,

S_1^2 is the variance of the probit mortality distribution,

S_2^2 is the variance of the dose distribution,

$$\therefore S_2^2 = 0.206^2 = 0.042$$

If the true probit slope is 2, then $S_1^2 = \left(\frac{1}{2}\right)^2 = 0.25$

$$\therefore \bar{b} = 1.85$$

Thus, the slope is reduced by 7.5%. The effect on probit slope is less as the true slope is reduced, being 4.4% at a slope of 1.5 and 2.0% at a slope of 1.0. Variation in dose caused by the dosage method therefore has a very small effect on overall dosage-response variability.

(10) Production and Counting of ^{32}P - and ^3H -Labelled Virus

Radio-labelled A. segetum NFV (English isolate) and P. brassicae GV were prepared using ^{32}P -orthophosphate or ^3H -Thymidine. Both radio-isotopes were supplied by the Radiochemical Centre, Amersham, U.K.

^{32}P -orthophosphate was supplied at 10 m Ci/ml in dilute Hcl (pH 2-3) and methyl- ^3H Thymidine at 1 m Ci/ml in 2% ethanol.

Radio-labelled virus was produced in the homologous host by feeding third to fourth instar larvae on diet with which the isotope had been incorporated. ^{32}P was incorporated by mixing with liquid diet, at 45°C, 0.5 ml isotope with 10 mls diet (sufficient for 50 larvae). ^3H -Thymidine was spread over the surface of pieces of diet, 10 μl isotope per larva being supplied. Virus inoculum was added at the same time as (A. segetum) or one day after (P. brassicae) feeding on labelled diet began.

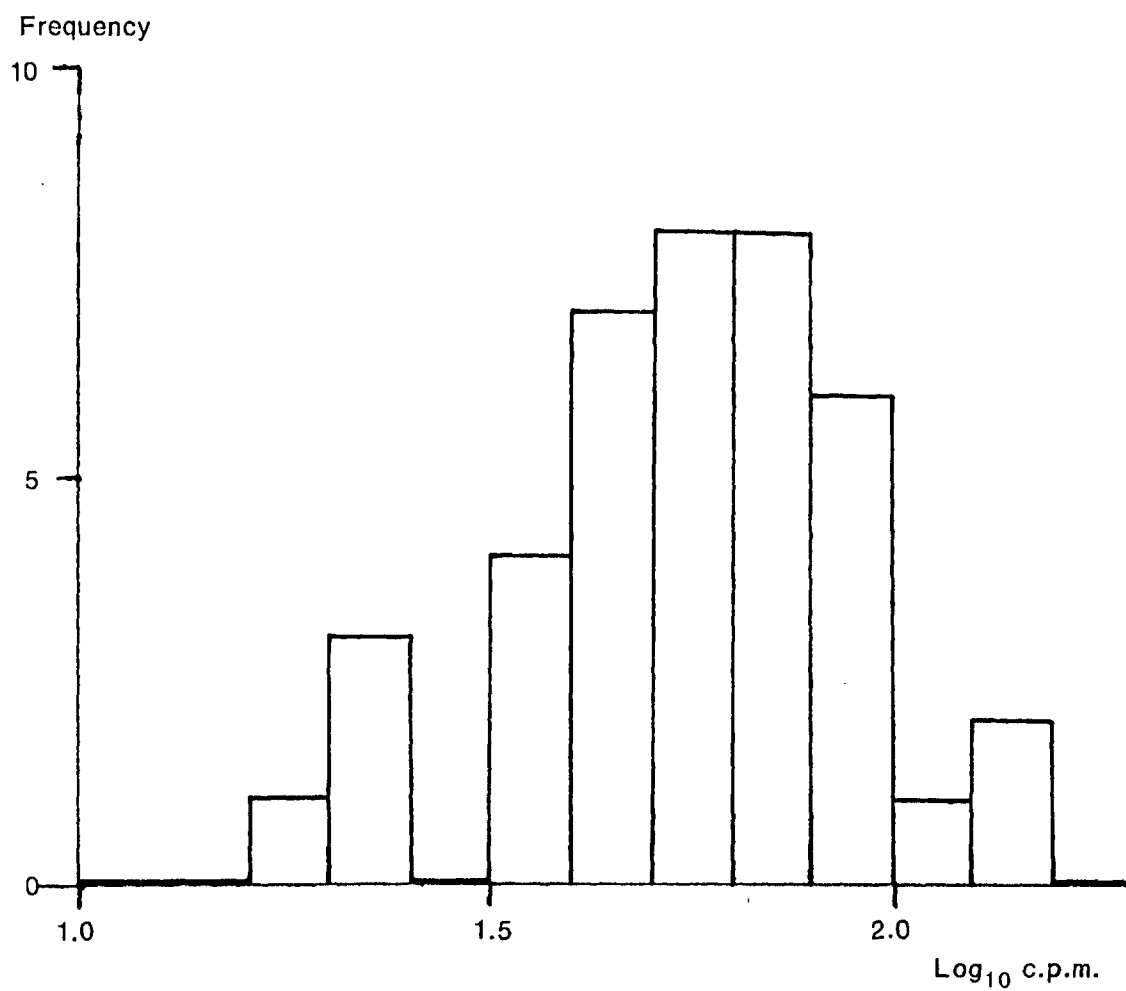


Figure 2.1. Histogram of bioassay dose distribution.

Radio-labelled virus was purified as described in Section 4(a). The level of radiocative emission was measured using a Tracerlab CM-200 liquid scintillation spectrometer. ^{32}P samples were dissolved or suspended in 20 mls 0.1% SDS and the level of Cerenkov radiation measured with the scintillation counter on tritium settings (Clausen, 1968). ^3H samples were dissolved or suspended in 1 ml H_2O and counted in an emulsion cocktail consisting of 10 mls of a 1:2 mixture of Triton X-100 and toluene (Patterson and Greene, 1965). The toluene contained 0.4% of 2,5 diphenyloxazole (PFO-primary scintillant) and 0.02% of 1,4-bis (2-(5-Phenyloxazolyl)) benzene (POPOP-secondary scintillant). Triton X-100 & toluene were supplied by BDH (U.K.) and the scintillants by Sigma (London). The 1:10 volume ratio of sample to cocktail produced a stable emulsion (Parle, 1976).

Samples were thoroughly mixed and left for one hour in the dark to stabilise before counting and to reduce photoluminescence effects. The level of quench was determined using internal standards.

The techniques used to investigate the fate of radio-labelled virus fed to larvae are described in Chapter 7.

CHAPTER 3

BIOCHEMICAL AND SEROLOGICAL ANALYSES OF THE VIRUS ISOLATES

<u>CONTENTS</u>	<u>PAGE</u>
(1) ELECTROPHORETIC PROFILES OF RESTRICTION ENDONUCLEASE DIGESTS OF BACULOVIRUS DNA'S	101
a) THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV	101
b) <u>M. BRASSICAE</u> NPV	104
c) <u>A. SEGETUM</u> GV	106
d) <u>P. GAMMA</u> sNPV AND mNPV	107
(2) ELECTROPHORETIC PROFILE OF <u>P. GAMMA</u> CPV GENOME SEGMENTS	111
(3) SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF STRUCTURAL POLYPEPTIDES FROM THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV AND <u>M. BRASSICAE</u> NPV	113
a) POLYHEDRINS	113
b) VIRUS PARTICLE STRUCTURAL POLYPEPTIDES	118
(4) SEROLOGICAL ANALYSIS OF THE BACULOVIRUSES	120
a) SPECIFICITY OF ANTISERA	120
b) ANTIGENIC RELATIONSHIPS BETWEEN THE BACULOVIRUSES, DETERMINED BY INDIRECT ELISA	124
(5) DISCUSSION	129

CHAPTER 3

BIOCHEMICAL AND SEROLOGICAL ANALYSES OF THE VIRUS ISOLATES

The biochemical and serological properties of the viruses were studied in order to provide criteria for identification of inocula and progeny viruses in cross-transmission experiments. Three European isolates of A. segetum NPV were examined in detail to determine their relatedness as a preliminary to a study of their host range and infectivity (Chapters 5 and 6). In addition an examination of serological relationships between all the baculovirus isolates was carried out to see how these related to their biological properties.

(1) Electrophoretic Profiles of Restriction Endonuclease Digests of Baculovirus DNA's

Profiles for the main baculoviruses studied are shown in Figures 3.1 to 3.5, with the exception of P. brassicae GV which has been recently characterised by Crook (1981(a)). The molecular weights of DNA fragments were calculated as the mean from two separate gel runs. Molarity of fragment bands was assessed visually.

a) Three European isolates of A. segetum NPV

The EcoRI and Hind III digest profiles of the three isolates are shown in Figures 3.1 and 3.2 respectively. The isolates from England (A. segetum (E) NPV) and France (A. segetum (F) NPV) appeared identical, while only one or two fragments shared similar mobilities with those of the Polish isolate (A. segetum (P) NPV). This indicated that at least 15% of the base pairs in A. segetum (P) NPV DNA differ from those in the DNA from the other isolates (Upholt, 1977). No similarity was found

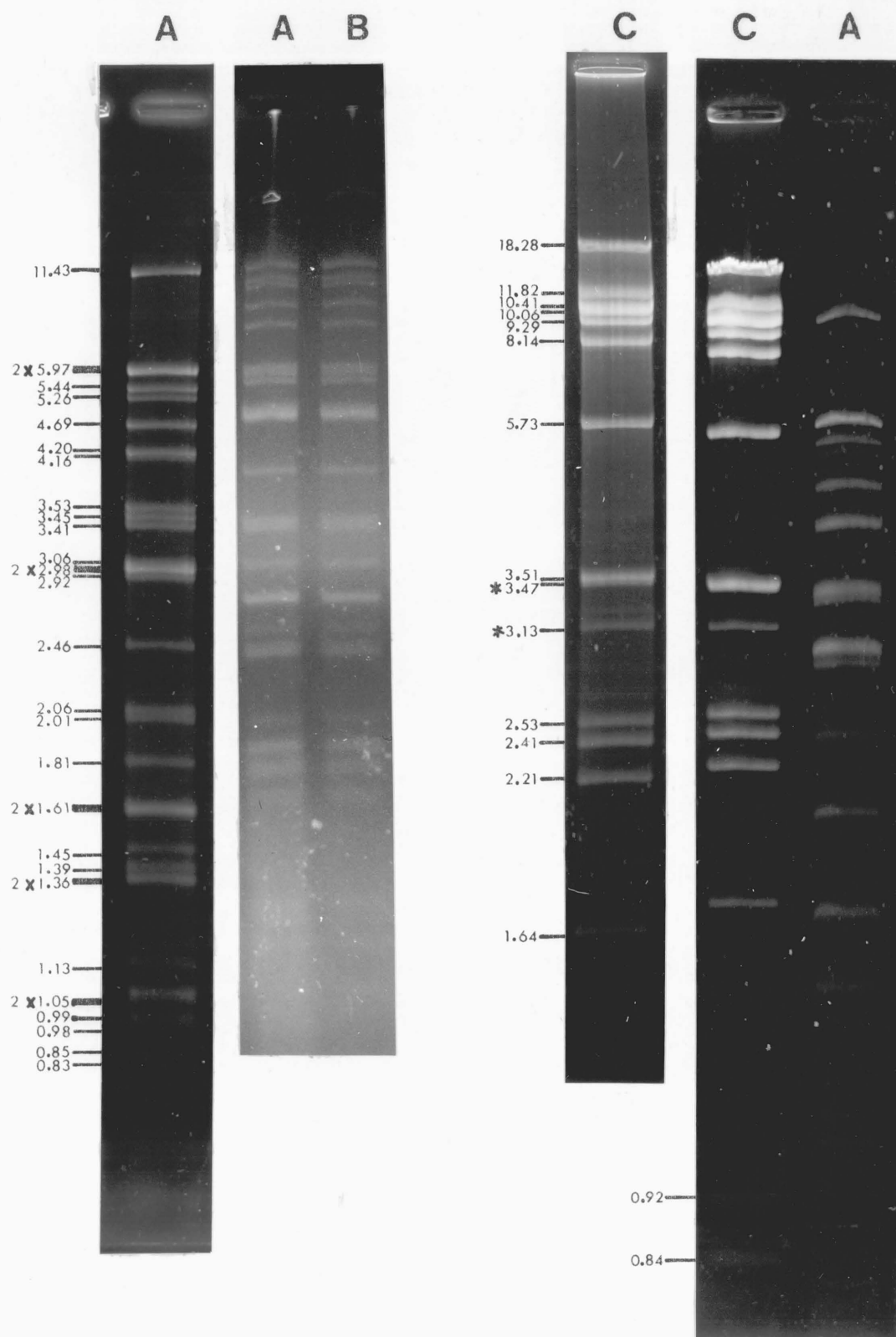


Figure 3.1.

Electrophoretic profiles of DNA, digested with EcoRI, from three European isolates of *A. segetum* NPV. Key:- A: *A. segetum* (E) NPV, B: *A. segetum* (F) NPV, C: *A. segetum* (P) NPV. Fragment molecular weights are given x 10⁻⁶
* : sub-molar fragment.

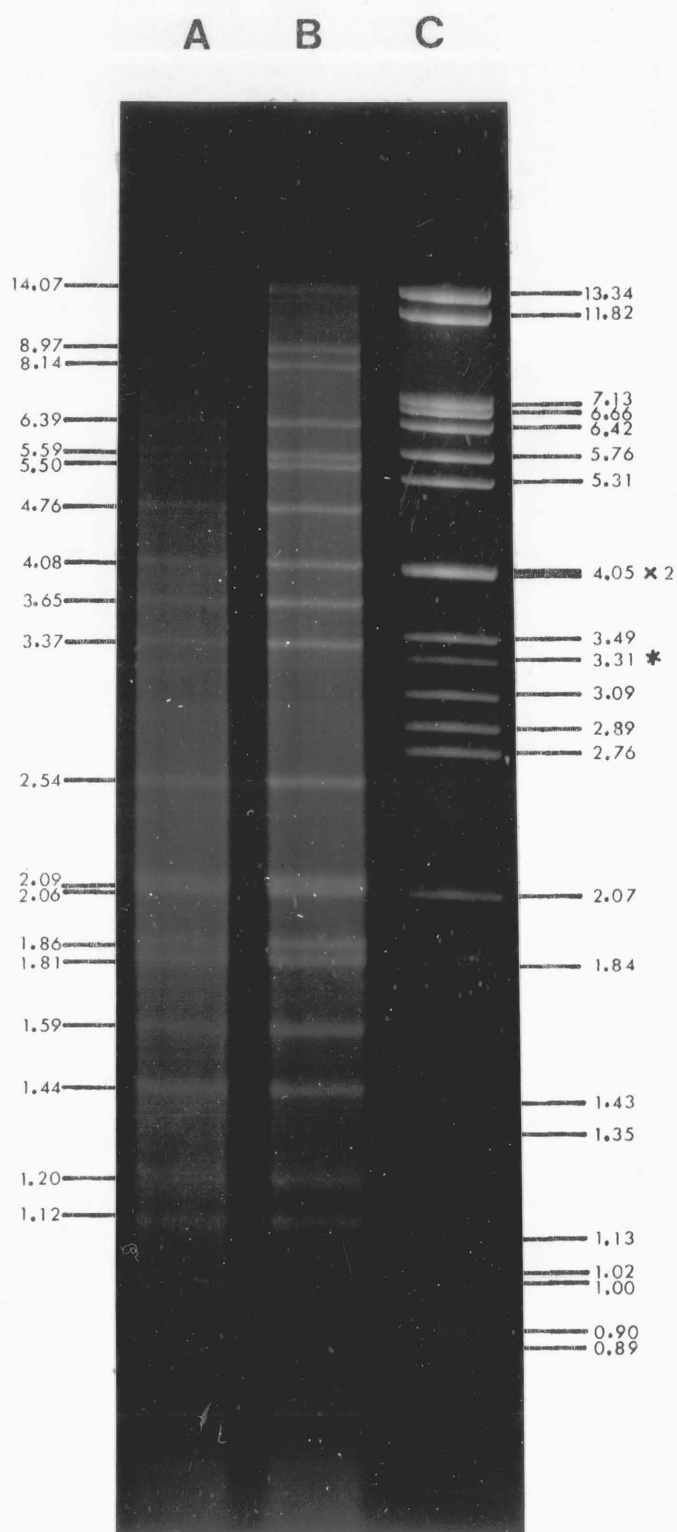


Figure 3.2.

Electrophoretic profiles of DNA, digested with *Hind* III, from three European isolates of *A. segetum* NPV. Key:- see Figure 3.1.

Note: fading of high molecular weight fragments in the *A. segetum* (E) NPV profile did not occur on other occasions.

between the profiles presented in Figures 3.1 and 3.2 and published profiles of other baculoviruses.

The occurrence of sub-molar bands in digests of A. segetum (P) NPV DNA with both enzymes (Figures 3.1, 3.2) suggested that genetic variants may exist in the virus population (Smith and Summers, 1978).

The total molecular weight of the fragments detected was as follows (ignoring sub-molar fragments):

	<u>EcoRI</u>	<u>Hind III</u>
English/French	93.45	80.23
Polish	87.79	88.40

These estimates of the genome molecular weight of the isolates will be underestimates, as fragments of molecular weight less than 0.8×10^6 have not been included. When the EcoRI digest of A. segetum (E) NPV DNA was separated on a 10% polyacrylamide gel approximately 10 extra fragments were detected, with molecular weights down to 0.35×10^6 (Appendix 1). It was difficult to accurately estimate the molecular weights of these fragments, but they totalled about 5×10^6 . The true genome molecular weight of both isolates appeared to be between 90 and 100×10^6 .

b) M. brassicae NPV

The EcoRI profile of this isolate is shown in Figure 3.3. The molecular weights of the fragments corresponded closely to those given by Vlak and Groner (1980) for the same (German) isolate. Several fragment bands had a greater intensity than adjacent bands, suggesting that they represented the co-migration of two different fragments (Figure 3.3). Those at molecular weights 6.53×10^6 and 2.44×10^6 were not recognised

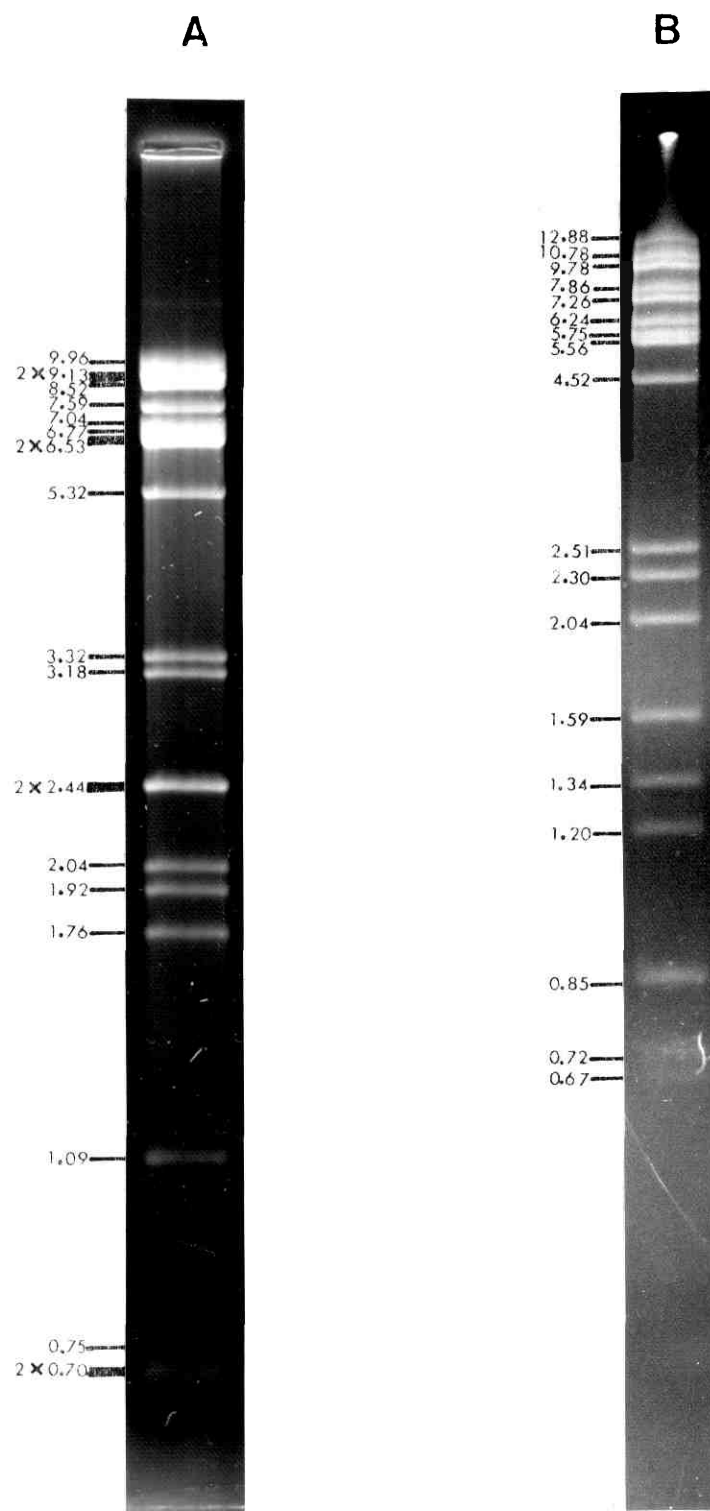


Figure 3.3.

Electrophoretic profiles of DNA, digested with EcoRI, from *M. brassicae* NPV(A) and *A. segetum* GV(B).

Molecular weights are given $\times 10^{-6}$

as double molarity bands by Vlak and Groner (1980), but were by Kelly and Brown (1980) who also studied this isolate. The latter authors also reported a fragment intermediate between the 6.53×10^6 and 5.32×10^6 fragments shown in Figure 3.3. This was not found by Vlak and Groner (1980), nor was it seen in the present study.

The total molecular weight of DNA fragments detected on 1% agarose gels was 96.86×10^6 . This was in close agreement with the figure of 98.97×10^6 derived from the estimate given by Vlak and Groner (1980) by adjustment for the two bands of double molarity which they missed. Kelly and Brown (1980) however, gave a lower molecular weight of 91.41×10^6 by EcoRI digestion (adjusted by subtraction of their extra band). This difference was due mainly to variation in the estimates of molecular weights of the largest fragments.

Approximately 6 extra low molecular weight fragments were found on polyacrylamide gel electrophoresis (Appendix 1). These had a total molecular weight of about 3×10^6 , giving a total genome molecular weight of approximately 100×10^6 . It appears that the estimate of 115×10^6 by Kelly and Brown (1980), using electron microscopy and reassociation kinetics, is an overestimate of the true genome molecular weight. When Jurkovicova et al. (1979) examined the genome of a closely related M. brassicae NPV isolate from Holland (Vlak and Groner, 1980) they estimated the molecular weight as 89×10^6 using electron microscopy and reassociation kinetics.

c) A. segetum GV

The EcoRI profile of DNA extracted from this isolate is shown in Figure 3.3. Restriction endonuclease profiles of this isolate did not appear similar to published EcoRI profiles of other viruses. The total

molecular weight of fragments $\geq 0.67 \times 10^6$ was 83.85×10^6 .

d) P. gamma sNPV and mNPV

An isolate of P. gamma NPV was obtained by M. E. Martignoni, while at the G.C.R.I. in 1972-73, from C. Vago (Station de Recherches de Pathologie Comparée, Saint-Christol-les-Alès, France). This isolate contained exclusively cubic polyhedra and was used as the inoculum for production of a larger batch in P. gamma for further analysis. The progeny virus from this infection consisted of cubic I.B.'s of the mNPV type when examined in thin section (Chapter 4). The electrophoretic profile of the EcoRI digest of DNA extracted from this isolate is shown in Figure 3.4. This profile was very similar to that of an isolate of Autographa californica mNPV obtained from C. F. Reichelderfer (University of Maryland, U.S.A.) and also shown in Figure 3.4. The P. gamma mNPV DNA digest had a submolar fragment at molecular weight 2.51×10^6 which was not found in the A. californica NPV isolate. This fragment was also found in an A. californica isolate examined by Smith and Summers (1978).

P. gamma NPV isolates were also obtained from the Institute of Virology (Oxford, U.K.), being isolates No.88 and No.287 from the virus collection maintained there. In neither case was the origin of the isolate recorded.

No.287 appeared to consist entirely of cubic I.B.'s of 1-4 μ m diameter when examined by phase contrast microscopy. When grown in P. gamma the progeny virus was multiply enveloped as determined by isopycnic sucrose gradient centrifugation. When the DNA was digested with EcoRI the electrophoretic profile was again similar to that of A. californica NPV DNA (Figure 3.4). Although the profile was faint, it was possible to see an extra band at 5.19×10^6 which was not present in

the A. californica profile. The NPV obtained from Galleria mellonella is closely related to A. californica NPV but also exhibits an extra band at this point in its EcoRI digest profile (Smith and Summers, 1979).

Isolate No.88 contained a high density of small polyhedral I.B.'s of around 1 μ m diameter, with some larger I.B.'s, when examined by phase contrast microscopy. When grown in P. gamma larvae, some corpses contained small polyhedral I.B.'s, some large cubic I.B.'s and others a mixture of these two I.B. forms. The corpses which apparently contained only small I.B.'s were pooled and the I.B.'s were purified. These appeared to contain only singly enveloped virus particles when examined by negative staining of partly dissolved polyhedra (Chapter 4). This virus batch was used as the inoculum for a further passage in P. gamma. A mixture of polyhedral types was again found in the progeny. Those corpses apparently containing only small I.B.'s were pooled. On this occasion an extra cycle of rate-zonal gradient centrifugation was used in the I.B. purification. A 30-80% (v/v) glycerol gradient was used at 3,000 r.p.m. for 40 minutes. These conditions had previously been shown to cause the larger, cubic, I.B.'s to pellet. The I.B.'s which had not pelleted were removed and purified. This preparation now appeared to consist entirely of the small I.B.'s when examined thoroughly by phase contrast microscopy. The electrophoretic profile of the EcoRI digest of DNA obtained from this isolate is shown in figure 3.5, together with the profile of a mixed batch of small and large I.B.'s. Although the latter DNA sample apparently did not digest quite to completion it was possible to detect bands of a profile similar to that of A. californica NPV mixed with that of P. gamma sNPV.

The P. gamma sNPV profile was not contaminated with A. californica bands. The faint submolar band at high molecular weight ($>15 \times 10^6$) was

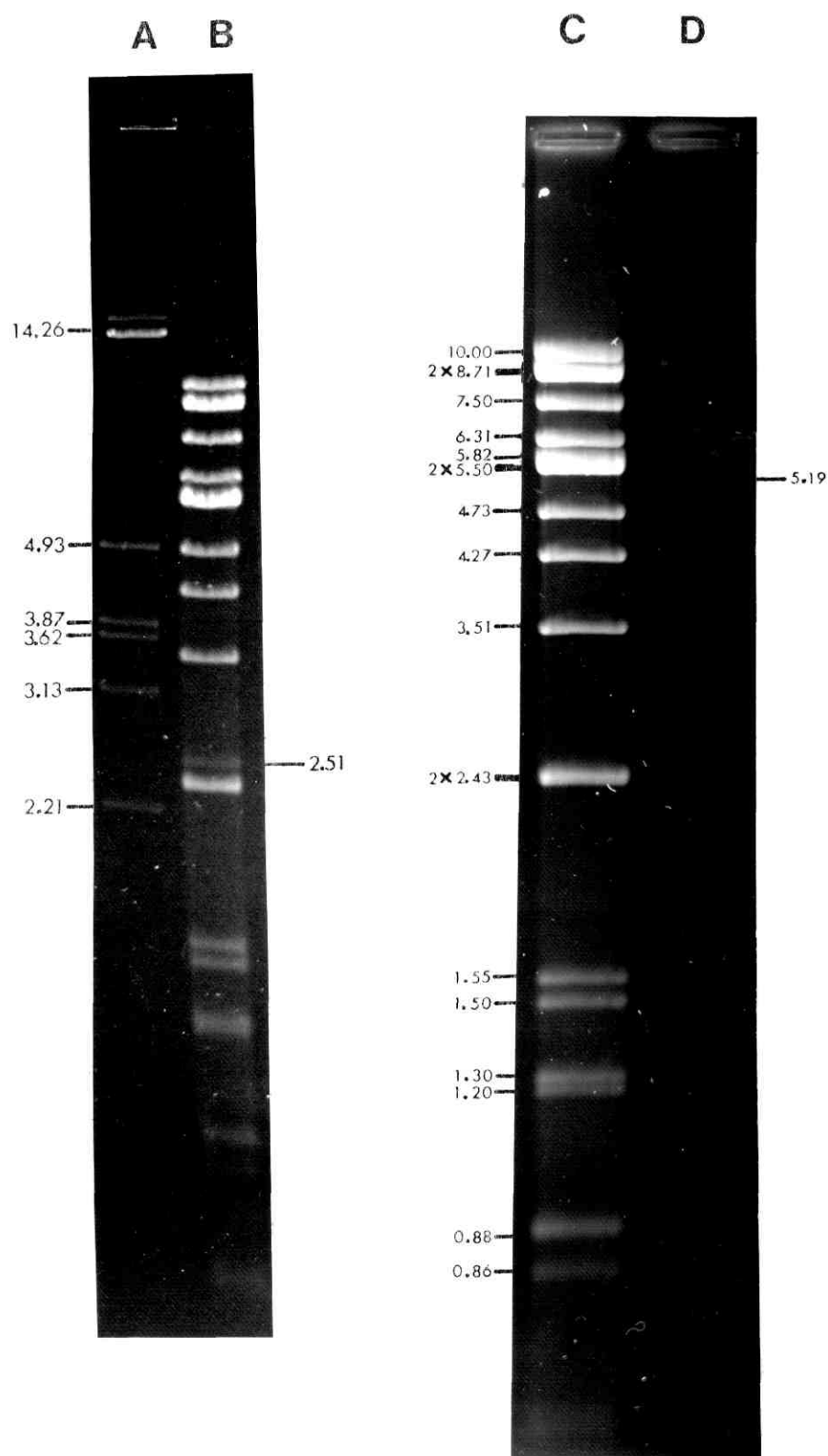


Figure 3.4. Electrophoretic profiles of DNA, digested with EcoRI, from *P. gamma* mNPV and *A. californica* NPV. Key:- A: λ DNA, B: *P. gamma* mNPV, from France, C: *A. californica* NPV, D: *P. gamma* mNPV, isolate 287 from Oxford.

Molecular weights are given $\times 10^{-6}$

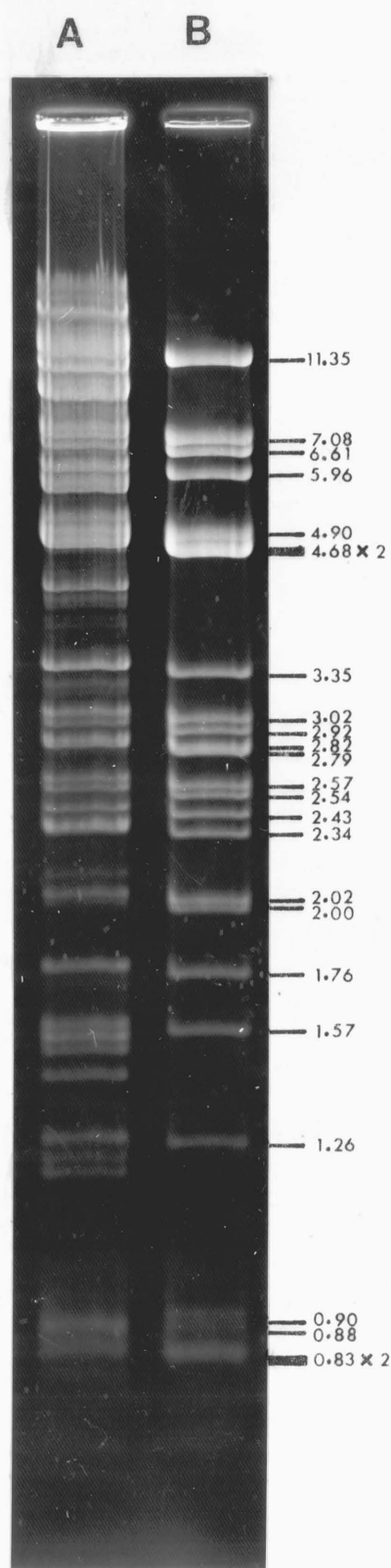


Figure 3.5. Electrophoretic profile of DNA, digested with EcoRI, from *P.gamma* sNPV. Key:- A: *P.gamma* sNPV + mNPV, B: *P.gamma* sNPV.

Molecular weights are given $\times 10^{-6}$

present on an adjacent digest profile of M. brassicae NPV (not shown) suggesting that it is an artifact. The sNPV profile showed no similarity with published EcoRI digest profiles of baculoviruses. The total molecular weight of the fragments detected (down to 0.83×10^6) was 82.09×10^6 .

This P. gamma sNPV isolate was used in all further work. No recurrence of larger cubic I.B.'s was found in infection tests, confirming the purity of the isolate.

It seems that P. gamma populations may be subject to at least two different NPV infections. One of these is an mNPV similar to members of the A. californica NPV virus complex. This is probably the isolate studied by Vago and Cayrol (1955) who described a cubic I.B. of 1-5 μ m. The sNPV of P. gamma is clearly distinct from this mNPV. It is not known whether this isolate is the same as the P. gamma sNPV used in infection tests by Harper (1976) and supplied to him by A. Burgerjon (I.N.R.A., La Minière, France).

(2) Electrophoretic Profile of P. gamma CPV Genome Segments

The profile of P. gamma CPV ds RNA segments on 3% acrylamide is shown in Figure 3.6. The molecular weights of segments were calculated by comparison with the Bombyx mori CPV segment molecular weights given by Fujii-Kawata et al. (1970) as standards. The profile of the B. mori CPV segments is also shown in Figure 3.6.

The P. gamma CPV profile was indistinguishable from that of a CPV extracted from Spodoptera exempta (Payne et al., 1977(b)). The molecular weights of ds RNA segments calculated for both CPV's were almost identical. It is probable that the P. gamma CPV, originally isolated in this species by M. E. Martignoni at the G.C.R.I., was transmitted to stocks of S. exempta

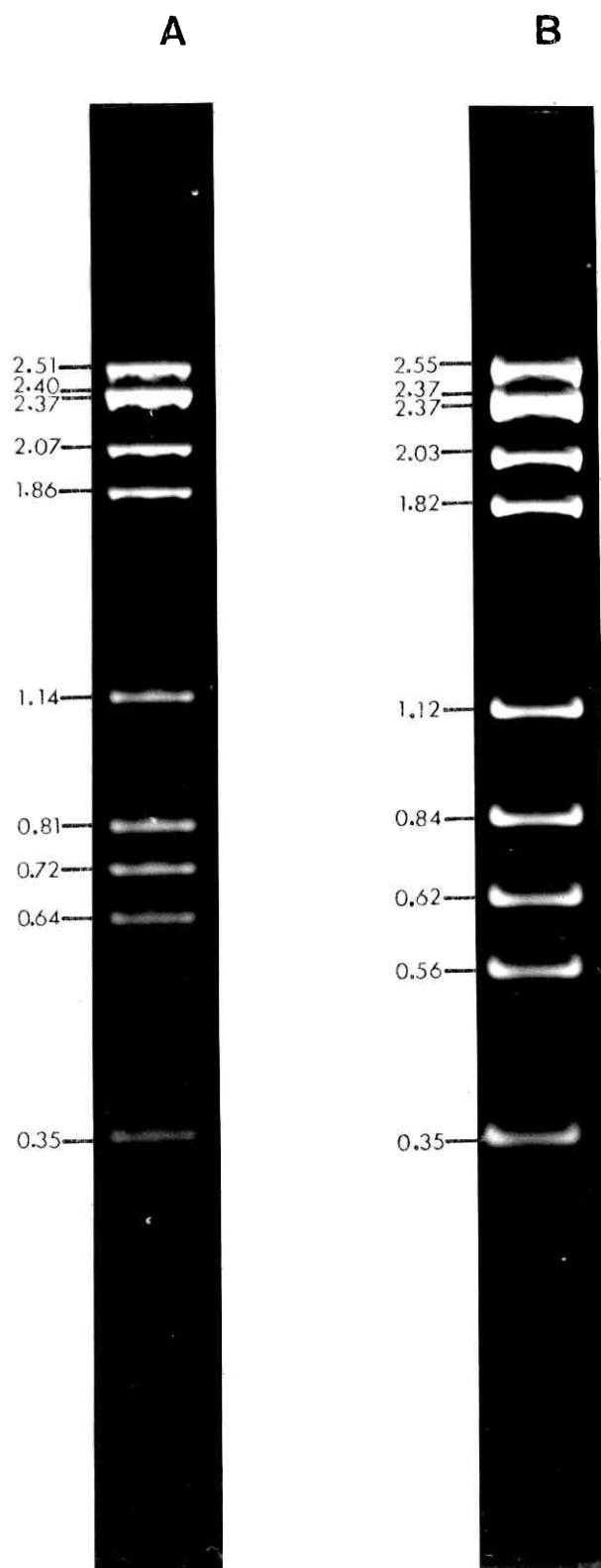


Figure 3.6. Electrophoretic profiles of *P.gamma* CPV(A) and *B.mori* CPV(B) genome segments on 3% acrylamide gel. Segment molecular weights are given $\times 10^{-6}$

which were being reared in the same department (Payne, pers. comm.). The segment profile of P. gamma CPV was similar to that of B. mori CPV and these viruses also appeared to be antigenically related (Payne et al., 1980). Close examination of the profiles showed too many differences to allow the P. gamma CPV isolate to be included in Type 1 (Payne and Rivers, 1976) and Payne et al. (1977(b)) classified the virus as type 12.

(3) SDS-Polyacrylamide Gel Electrophoresis of Structural Polypeptides from Three European Isolates of A. segetum NPV and M. brassicae NPV

The structural polypeptides of the three European A. segetum mNPV isolates were compared in order to confirm that the French and English isolates were identical, and to reveal any similarities between these and the Polish isolate. M. brassicae mNPV was included in the comparison. The electrophoretic profiles of polypeptides from this virus isolate have been previously studied (Kelly and Brown, 1980; Maskos and Miltenburger, 1981).

Molecular weight estimates calculated below were usually based on the results of two separate gel runs.

a) Polyhedrins

Slight differences were found in the mobility of the polyhedrins (Figure 3.7). The following molecular weight estimates were made for polyhedrins:

<u>M. brassicae</u> NPV	29,300
<u>A. segetum</u> (E) NPV	28,200
<u>A. segetum</u> (F) NPV	28,200
<u>A. segetum</u> (P) NPV	28,500

A. segetum (E) NPV and A. segetum (F) NPV polyhedrins appeared to have identical mobility. A. segetum (P) NPV polyhedrin may have a slightly lower mobility (Figure 3.7) but it was not possible to separate its

polyhedrin band from that of A. segetum (E) NPV by co-electrophoresis (not shown). M. brassicae NPV polyhedrin could be separated from A. segetum (E) NPV polyhedrin by this method (Figure 3.8).

The M. brassicae NPV polyhedrin molecular weight estimate was slightly higher than that published by Kelly and Brown (1980) of 28,000 and Maskos and Miltenburger (1981) of 28,200. It was lower than the estimate of 30,000 by Croizier and Croizier (1977), who also estimated the molecular weight of A. segetum (F) NPV polyhedrin as 29,400.

The use of a slab gel system in the present study allowed direct comparison of mobilities of viral polypeptides and molecular weight standards, which should increase the accuracy of the comparative estimates. M. brassicae NPV polyhedrin had a lower mobility than carboxypeptidase a (Figure 3.8) indicating a molecular weight greater than 29,000.

In Figure 3.7 additional polypeptides were resolved at molecular weight 25,000-26,000. These were breakdown products generated by the action of endogenous alkaline protease on polyhedrin. In this case the degradation was limited by dissolving polyhedra at 4°C. When dissolution was carried out at room temperature, extensive polyhedrin breakdown occurred (Figure 3.9). This figure shows the results of an examination of alkaline protease activity in M. brassicae NPV and A. segetum (E) NPV. Tracks A, B, C and D show the polypeptide profile of whole polyhedra, dissolved for 20 minutes at room temperature by addition of an equal volume of 0.1 M Na₂CO₃ to the polyhedral suspension. In the case of tracks A and C the polyhedral suspension was heated to 70°C for 30 minutes prior to dissolution to inactivate any enzymes present (Summers and Smith, 1976). Tracks E and F show the profiles of polyhedrins purified after polyhedra had been dissolved for three hours at room temperature without heat treatment.

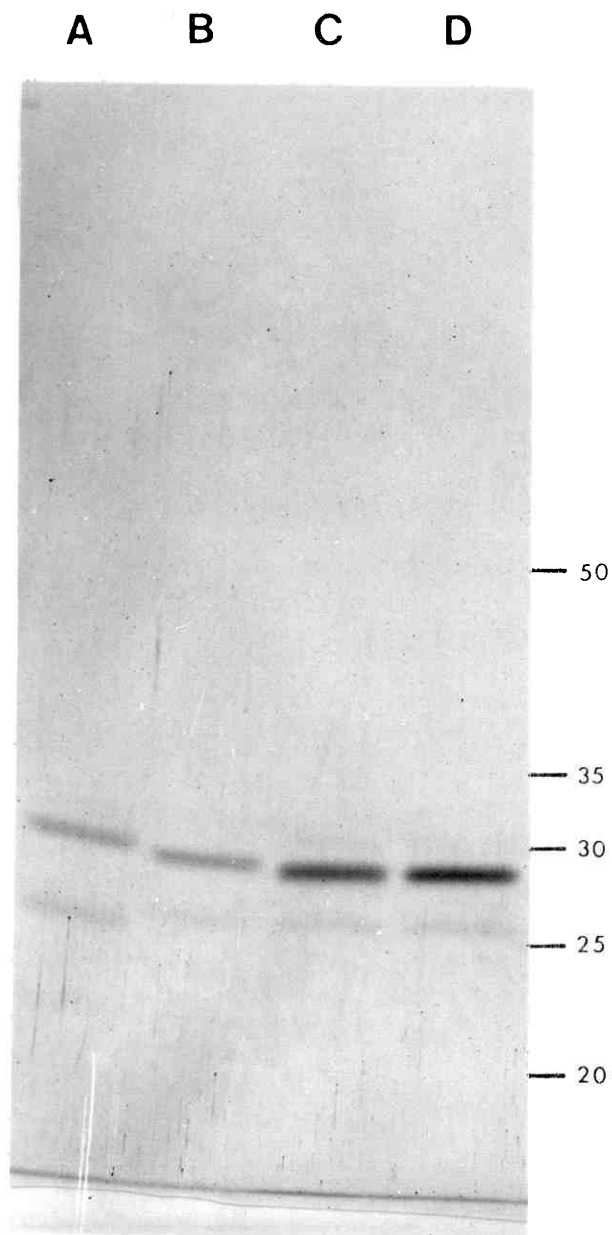


Figure 3.7. Polyacrylamide gel electrophoresis of polyhedrins. Key:- A: *M. brassicae* NPV, B: *A. segetum* (P) NPV, C: *A. segetum* (F) NPV, D: *A. segetum* (E) NPV. Molecular weights are given $\times 10^{-3}$

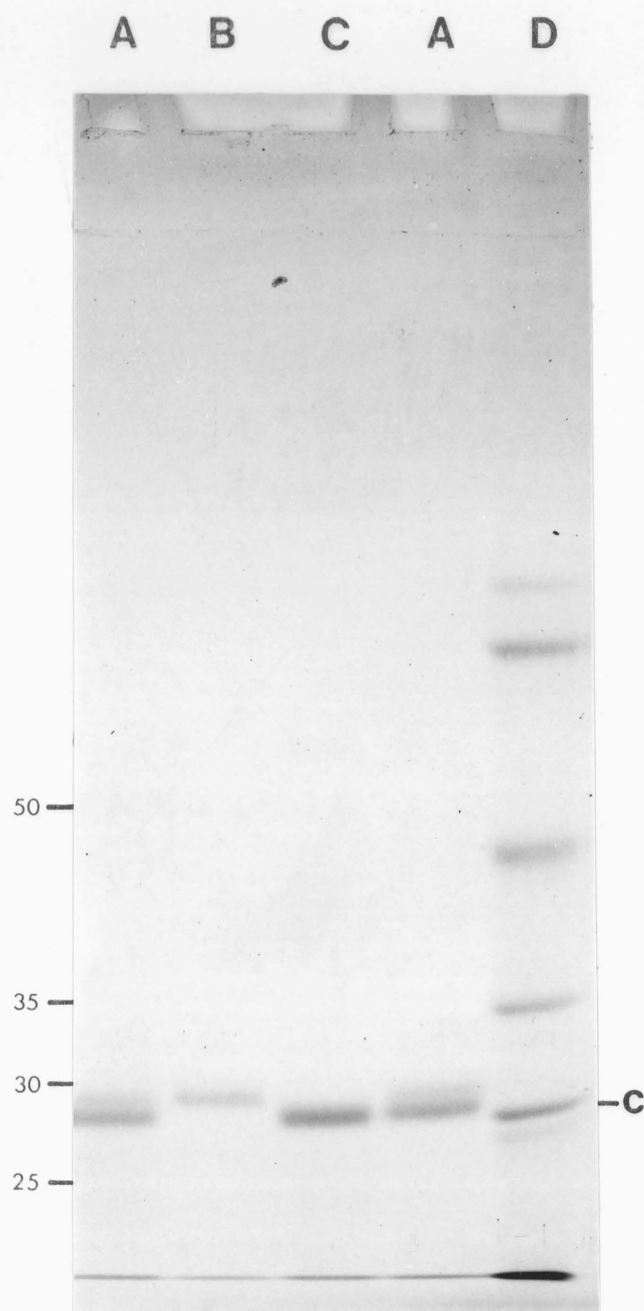


Figure 3.8. Co-electrophoresis of *M. brassicae* NPV and *A. segetum* (E)NPV polyhedrins.
 Key:- A: *M. brassicae* NPV + *A. segetum* (E) NPV, B: *M. brassicae* NPV,
 C: *A. segetum* (E) NPV, D: Molecular weight standards (c = carboxypeptidase a).

Molecular weights are given $\times 10^{-3}$

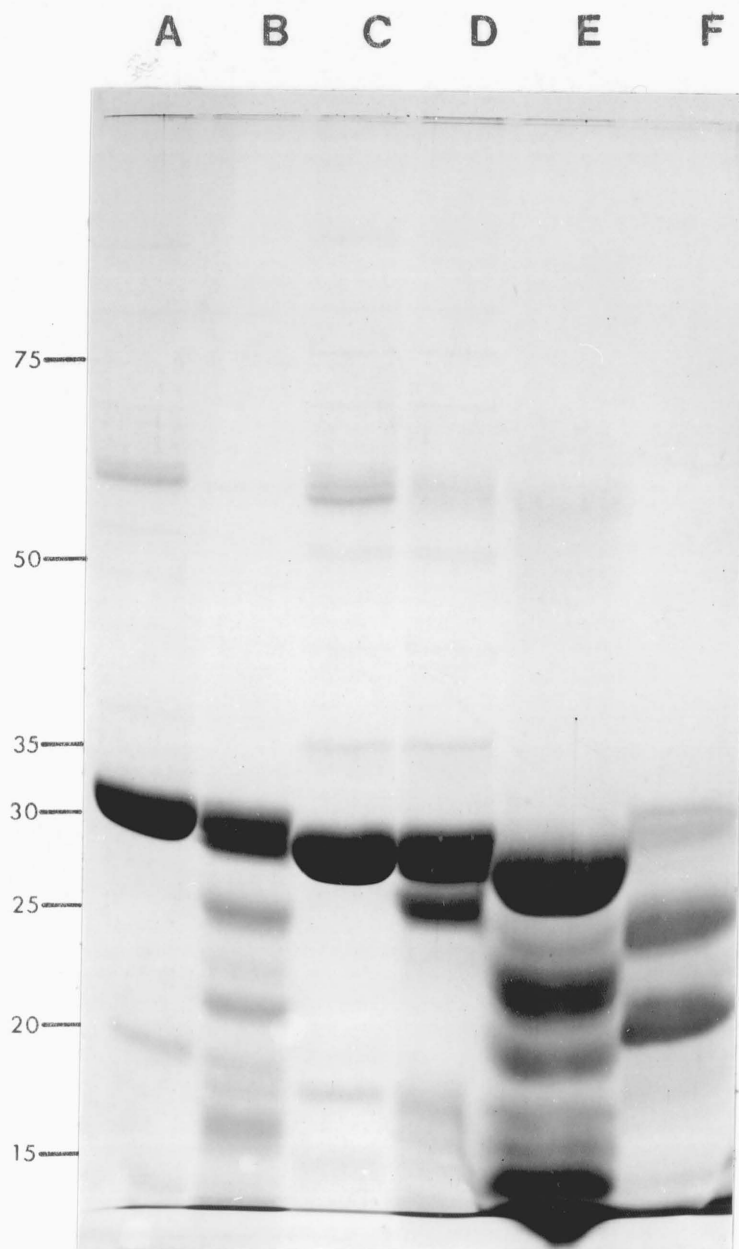


Figure 3.9. Electrophoresis of polyhedrins with varying degrees of alkaline protease breakdown. For description see text (Section 3(a)).

Molecular weights are given $\times 10^{-3}$.

Heat treatment considerably reduced polyhedrin breakdown (Figure 3.9, tracks A and C), although polypeptides with molecular weights of 20,000 (M. brassicae NPV) and 18,000 (A. segetum (E) NPV) were produced which did not correspond to virus particle polypeptides (Section 3(b)). The heat treated samples exhibited bands at molecular weights 58,900 (M. brassicae NPV) and 56,900 (A. segetum (E) NPV). These values were very close to twice the respective polyhedrin molecular weights and probably represented polyhedrin dimers. Other faint bands in these tracks corresponded to virus particle polypeptides.

Distinctive patterns of polyhedrin breakdown were produced when polyhedra were dissolved without heat treatment (tracks B, D, E and F).

b) Virus particle structural polypeptides

The electrophoretic mobilities and molecular weights of virus particle polypeptides from the three A. segetum NPV isolates and M. brassicae NPV are shown in Figure 3.10.

The bands marked with an asterisk appeared to be polyhedrin contaminating the preparation rather than virus particle polypeptides. They had the same molecular weight as polyhedrin and were variable in intensity, being absent from some preparations.

Virus particle polypeptides of molecular weight approximately 13,000 were detected in preparations from M. brassicae NPV and A. segetum (E) NPV. The other A. segetum isolates were not electrophoresed for periods short enough for resolution of bands below molecular weight 16,000.

Approximately 22 polypeptides were detected in the M. brassicae NPV virus particles. The relative mobilities of these polypeptides showed a similarity to the results published by Kelly and Brown (1980) and

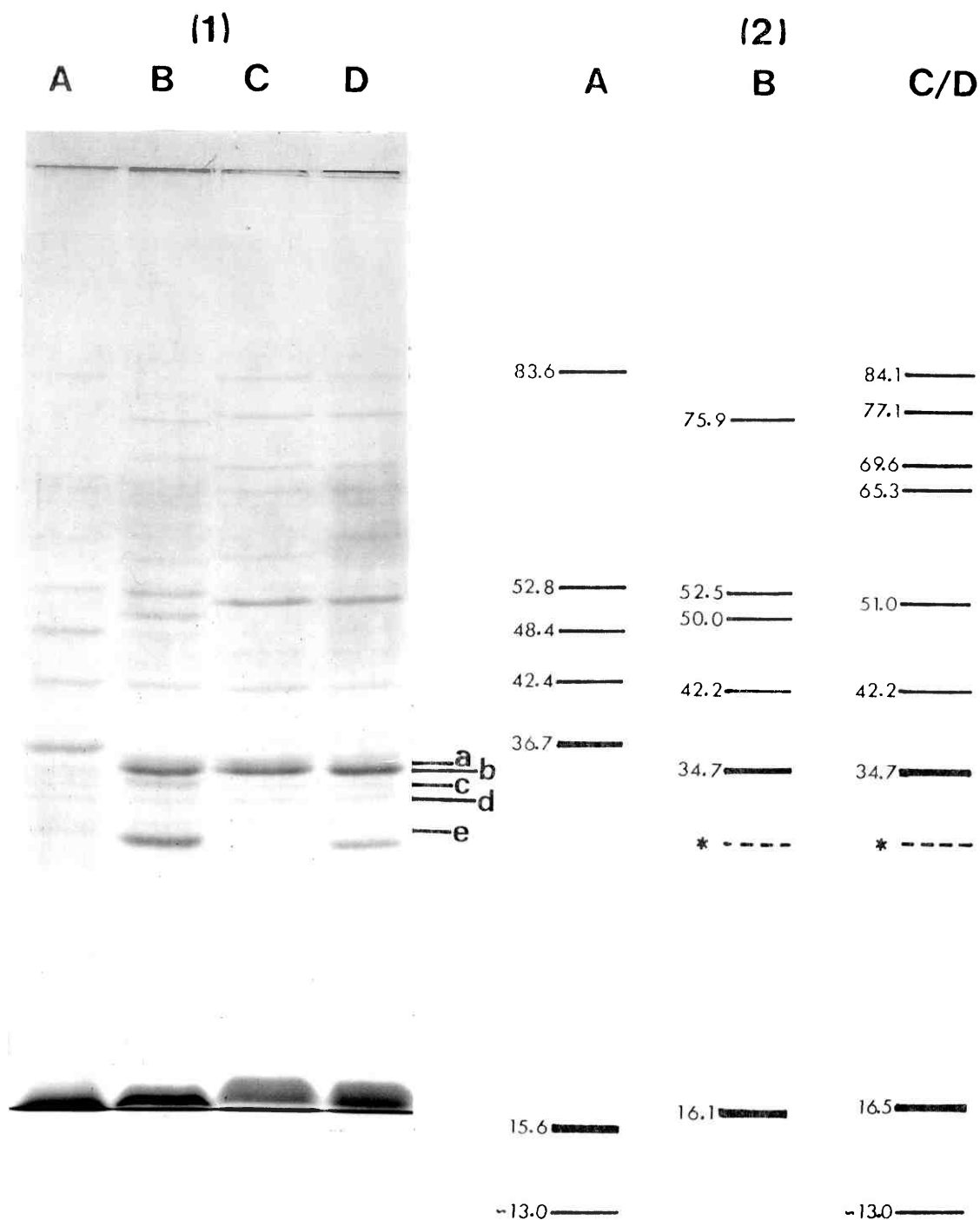


Figure 3.10. Electrophoresis of virus particle structural polypeptides from *M. brassicae* NPV and 3 European isolates of *A. segetum* NPV. (1) Gel photograph, (2) Diagram showing major polypeptide molecular weights $\times 10^{-3}$. Key:- A: *M. brassicae* NPV, B: *A. segetum* (P) NPV, C: *A. segetum* (F) NPV, D: *A. segetum* (E) NPV. See text for explanation of symbols.

Maskos and Miltenburger (1981) although the molecular weights presented differed somewhat in all three studies. The relative intensity of the bands also varied from study to study so that, although the same isolate was used, it was not possible to use the profiles for confident virus identification.

A. segetum (E) NPV and A. segetum (F) NPV had almost identical profiles, with approximately 23 polypeptide bands. A small difference in two minor bands is arrowed in Figure 3.10 but was not consistently found. A. segetum (P) NPV virus particles contained approximately 20 polypeptides of which at least five (a-e in Figure 3.10) appeared to have the same mobility as polypeptides from the other A. segetum NPV isolates. Thus although A. segetum (P) NPV could be clearly distinguished from the other isolates it may share some similar virus particle polypeptides. The location of these polypeptides within the virus particle was not determined.

No similarity was detected between the profiles of M. brassicae NPV virus particle polypeptides and those of the A. segetum NPV isolates.

(4) Serological Analysis of the Baculoviruses

a) Specificity of antisera

A study of antisera specificity was carried out to see if serological testing could be used to identify virus isolates. This study used antisera to whole polyhedra and polyhedral components of A. segetum (E) NPV and M. brassicae NPV. The reactions of these antisera with dissolved I.B.'s of six virus isolates were tested by immunodiffusion. In addition, the M. brassicae NPV antisera were tested for specificity using the double-antibody sandwich ELISA.

The following isolates were used as antigens in the immunodiffusion tests: A. segetum (E) NPV; A. segetum (F) NPV; A. segetum GV; M. brassicae NPV (isolate described in Table 2); M. brassicae NPV (Dutch isolate obtained from J. M. Vlak; Dept. Virology, Wageningen, Holland); A. californica NPV (origin described in Section 1(d)).

Antisera showed various degrees of cross-reaction with heterologous NPV's but very few reacted with A. segetum GV. Some of the results are shown in Figure 3.11. The most specific antiserum was that prepared to A. segetum (E) NPV whole polyhedra. This formed a single precipitin line with dissolved polyhedra of A. segetum (E) NPV and A. segetum (F) NPV, showing a reaction of identity between these isolates. M. brassicae virus particle antiserum reacted with M. brassicae NPV and A. segetum (E) NPV and very weakly with M. brassicae (Dutch) NPV and A. segetum (F) NPV. Antisera to M. brassicae NPV I.B.'s and to A. segetum (E) NPV polyhedrin showed cross-reactions with all NPV's and the latter antiserum also reacted weakly with A. segetum GV (Figure 3.11).

The extensive cross-reactions shown by antisera in immunodiffusion tests indicated that this technique would be of limited value for virus identification.

Double antibody sandwich ELISA is the most specific of ELISA methods in general use (Crook and Payne, 1980). The level of cross-reaction of M. brassicae NPV virus particle and polyhedrin antisera with A. segetum (E) NPV dissolved polyhedra was compared with their reaction with M. brassicae NPV dissolved polyhedra using this technique. The results (Figure 3.12) demonstrate significant cross-reaction, although the homologous virus reacted more strongly. It is not clear why the reaction with A. segetum NPV was diminished at higher antigen concentrations

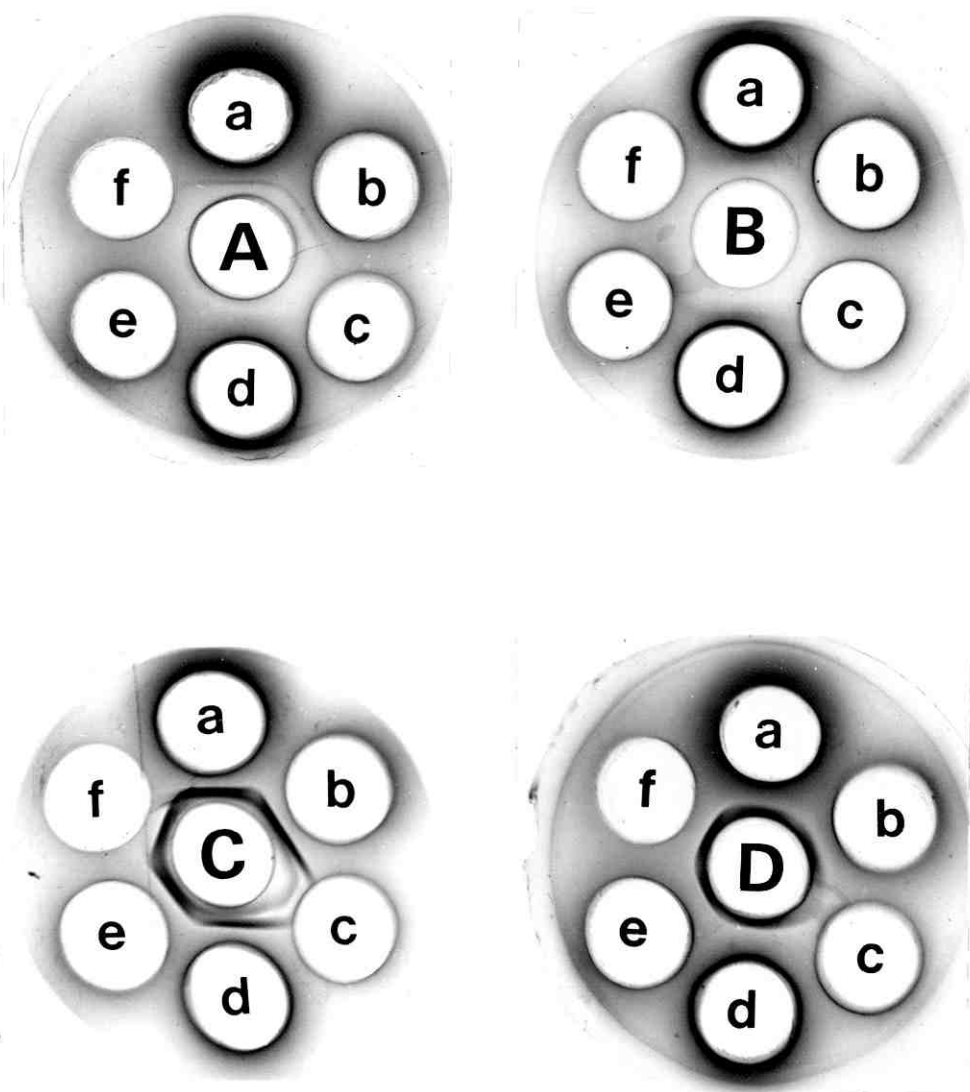


Figure 3.11. Immunodiffusion tests of antisera specificity.

Key to antisera:-

- A: *A. segetum* (E) whole polyhedra antiserum.
- B: *M. brassicae* virus particle antiserum.
- C: *A. segetum* (E) polyhedrin antiserum.
- D: *M. brassicae* whole polyhedra antiserum.

Key to antigens :-

- a: *A. segetum* (E) NPV.
- b: *A. segetum* (F) NPV.
- c: *A. segetum* GV.
- d: *M. brassicae* NPV (German isolate).
- e: *M. brassicae* NPV (Dutch isolate).
- f: *A. californica* NPV.

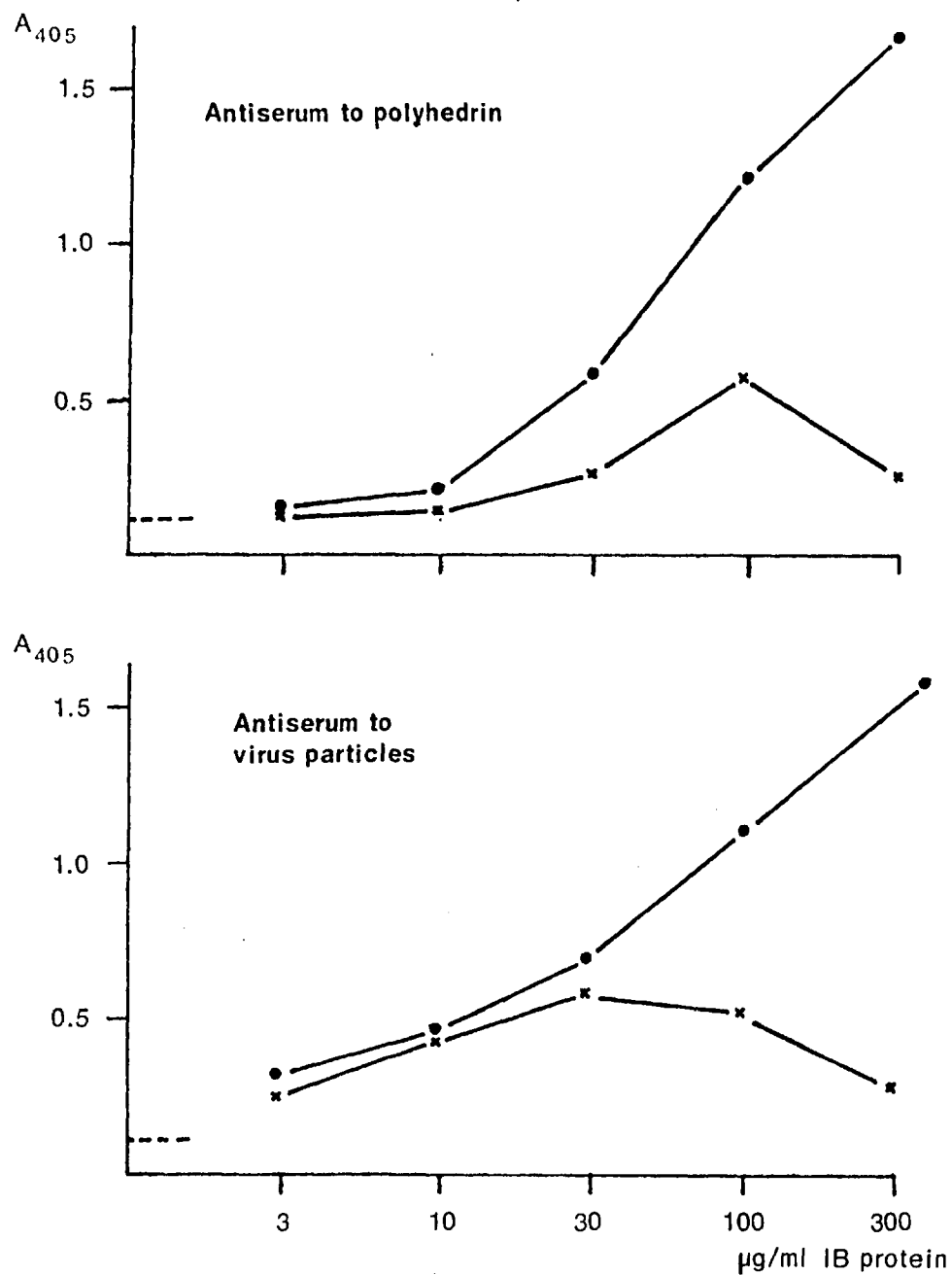


Figure 3.12. Reaction of *M. brassicae* NPV antisera with dissolved NPV IB's tested by double antibody sandwich ELISA.

antigens : ● *M. brassicae* NPV
 x *A. segetum* NPV
 ---- Background absorbance

in both cases, although a similar phenomenon was found by Crook and Payne (1980).

These ELISA results confirmed that serological methods were not suitable for discrimination of the viruses used in this study, due to varying levels of cross-reaction of antisera with heterologous antigens.

b) Antigenic relationships between the baculoviruses determined by indirect ELISA

While Section (a) demonstrates that serological methods were not the most appropriate for identification of the virus isolates, the phenomenon of antigenic cross-reaction permitted study of relationships between the viruses. This was based on the assumption that the degree of antigenic cross-reaction is related to phylogeny (Upholt, 1977; Rohrmann et al., 1981).

In this study the indirect ELISA was used to analyse these relationships. This ELISA method is less specific than the double antibody sandwich technique (Crook and Payne, 1980). It has the advantage that tests using different antisera can be performed with a standard conjugate preparation.

Three series of experiments were carried out, using dissolved I.B.'s as antigens:

(i) Compared M. brassicae NPV, A. segetum (E) NPV and A. segetum GV using antisera to virus particles, polyhedrin and polyhedra of M. brassicae NPV and A. segetum (E) NPV (Figure 3.13).

(ii) Compared all the main baculovirus isolates including the three A. segetum NPV isolates. Antisera to A. segetum

(E) NPV polyhedrin and virus particles were used
(Figure 3.14).

(iii) Compared the main baculovirus isolates except A. segetum
(F) NPV and A. segetum (P) NPV, using antisera to
M. brassicae NPV virus particles and A. segetum GV
capsules (Figure 3.15).

The results presented in Figure 3.13 demonstrated considerable cross-reaction with heterologous NPV antigens. This confirmed the pattern found in section 4(a). There was some cross-reaction with A. segetum GV antigens, but this was at a lower level. A. segetum (E) NPV virus particle antiserum was the most specific, while most other antisera showed similar levels of reaction with both NPV's. A. segetum (E) NPV antisera to whole polyhedra reacted more strongly with M. brassicae NPV than with the homologous virus. This suggests that the antigens responsible for cross-reaction with this antisera are present at a higher concentration in M. brassicae NPV I.B.'s than in A. segetum NPV I.B.'s. This result contrasted with the gel diffusion test using this antiserum (Section 4(a)), demonstrating the influence of test method on the results of serological tests.

When antisera to A. segetum (E) NPV virus particles and polyhedrin were tested against all the main baculoviruses of this study three levels of reaction were found (Figure 3.14). A. segetum (E) NPV and A. segetum (F) NPV reacted with almost equal intensity, strengthening the belief that they are identical isolates. A. segetum (P) NPV reacted to the same extent as M. brassicae NPV and these both reacted less than the other two A. segetum NPV isolates. A. segetum GV and P. brassicae GV exhibited the least reaction, although A. segetum GV reacted more strongly than P. brassicae

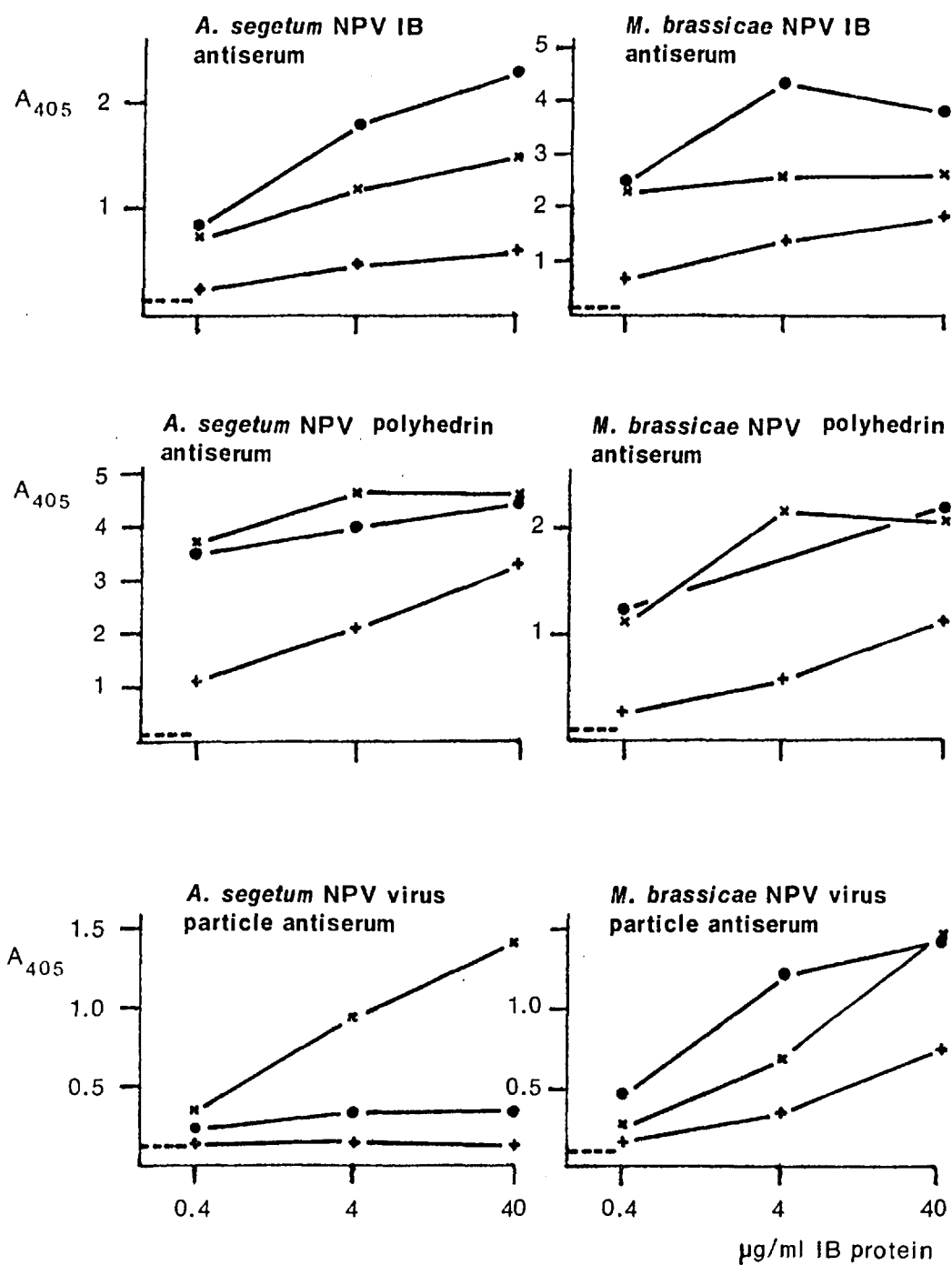


Figure 3.13. Reaction of NPV antisera with dissolved NPV and GV IB's, tested by indirect ELISA.

antigens : ● *M. brassicae* NPV
 x *A. segetum* NPV
 + *A. segetum* GV
 ---- Background absorbance

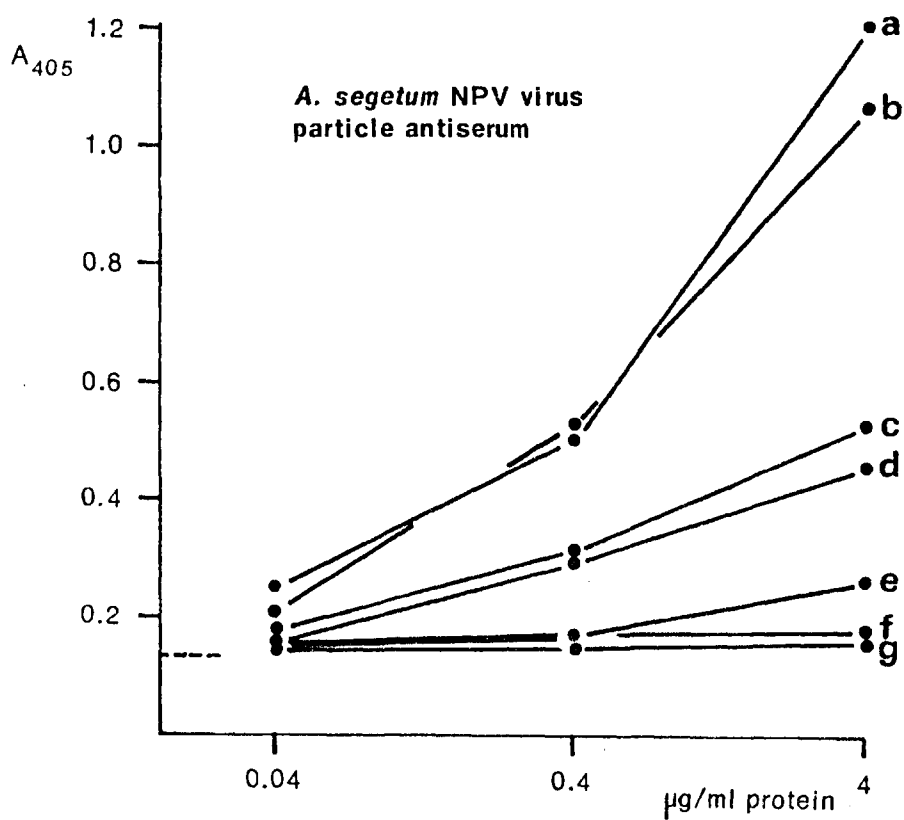
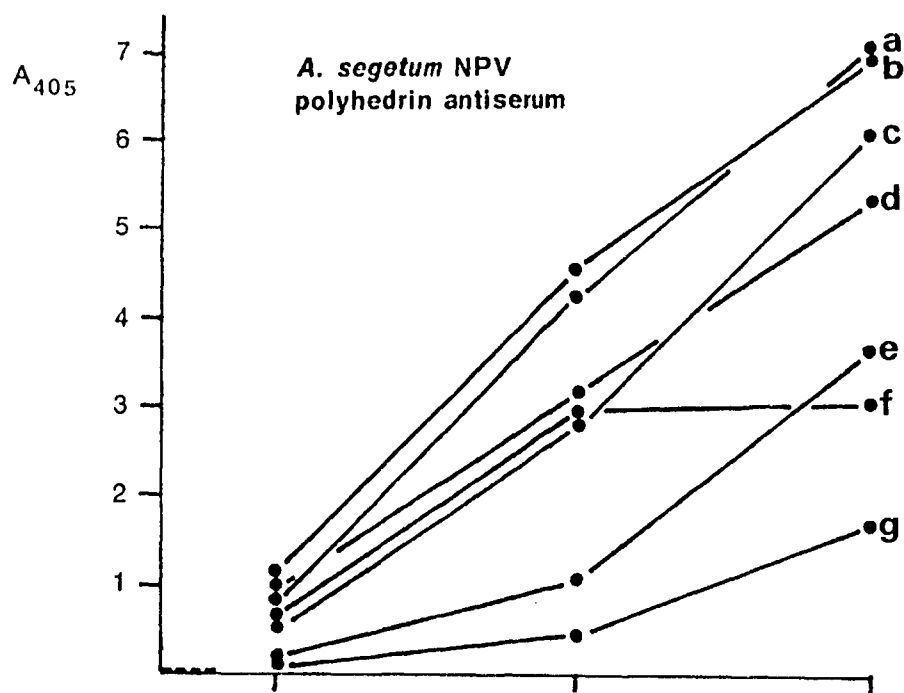


Figure 3.14. Reaction of *A. segetum* (E) NPV antisera with dissolved NPV and GV IB's, tested by indirect ELISA.
 antigen key : a, *A. segetum* (E) NPV; b, *A. segetum* (F) NPV;
 c, *A. segetum* (P) NPV; d, *M. brassicae* NPV;
 e, *A. segetum* GV; f, *P. gamma* SNPV;
 g, *P. brassicae* GV.

----- Background absorbance

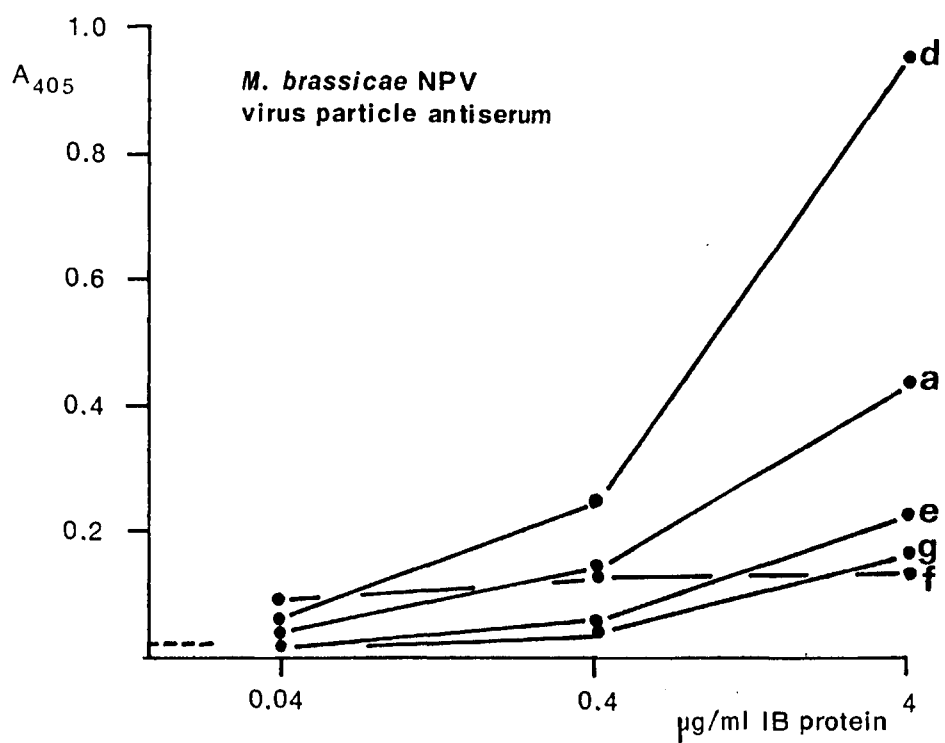
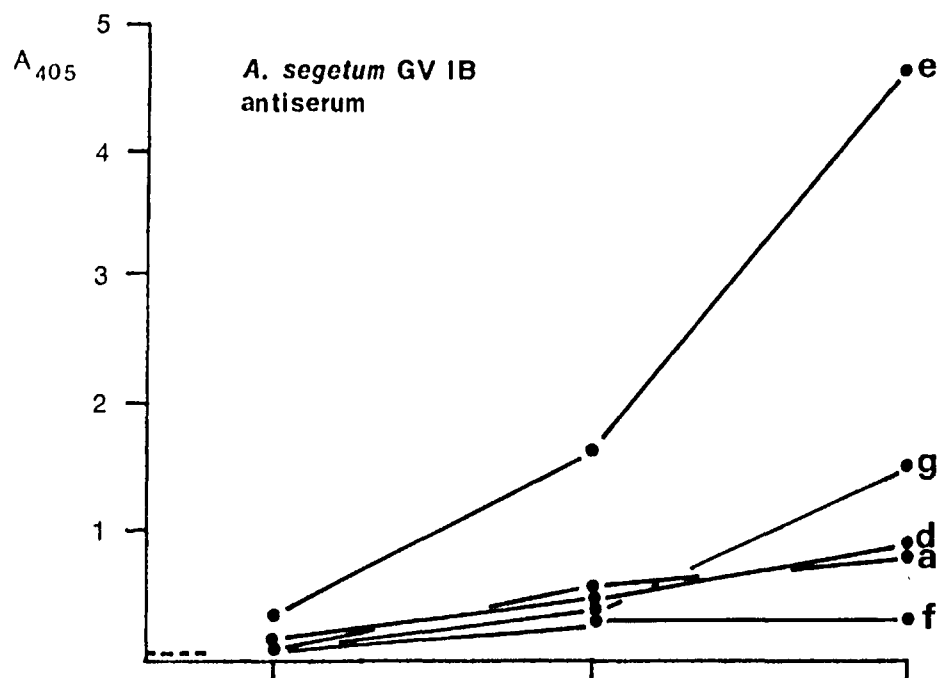


Figure 3.15. Reaction of NPV and GV antisera with dissolved NPV and GV IB's, tested by indirect ELISA.

key: see Figure 3.14.

GV with both antisera. The P. gamma sNPV reaction was intermediate to those of A. segetum (P) NPV and A. segetum GV with antiserum to A. segetum (E) NPV polyhedrin, but was similar to those of the GV's with the virus particle antiserum.

A similar relationship between mNPV, GV and sNPV was found when five viruses were tested with A. segetum GV capsule antiserum and M. brassicae NPV virus particle antiserum (Figure 3.15). A. segetum GV capsule antiserum was fairly specific, showing only weak reactions with heterologous antigens. Of these cross-reactions, that with P. brassicae GV was strongest, the mNPV's showing a weaker reaction and P. gamma sNPV a very weak cross-reaction. The reactions of M. brassicae NPV virus particle antiserum with the two mNPV's and A. segetum GV are discussed above. P. brassicae GV exhibited a similar reaction to that of A. segetum GV while the P. gamma sNPV reaction was intermediate between the mNPV's and GV's.

(5) Discussion

Of the techniques used for identification of baculovirus isolates, the electrophoresis of restriction enzyme fragments of viral DNA was the most suitable. It was possible to compare the electrophoretic profiles with those published by other laboratories and so confirm if the same isolate was involved in a particular study. The electrophoretic profile of ds RNA genome segments was similarly useful in the identification of P. gamma CPV and its comparison with other CPV isolates.

Electrophoresis of virus particle polypeptides was a useful technique for distinguishing baculovirus isolates in this laboratory. However the profiles produced were not identical with those found in other

laboratories using the same isolate. This technique was not as valuable as electrophoresis of restriction enzyme digest fragments for identification of the baculovirus isolates.

Antisera to whole I.B.'s or I.B. components exhibited varying degrees of cross-reaction with heterologous antigens. This limited their use in identifying viruses, but made them valuable for studying relationships between isolates.

In the present study A. segetum (E) NPV and A. segetum (F) NPV could not be distinguished by any of these three techniques and are probably identical isolates. A. segetum (P) NPV appeared to share some virus particle polypeptides of the same molecular weight with the other A. segetum NPV isolates but was clearly different from these isolates in terms of polypeptide and DNA fragment electrophoretic profiles. Serological analysis failed to show any close relationship between this isolate and the other two. The evidence suggests that A. segetum (P) NPV is a distinct virus rather than a variant of A. segetum (E) NPV. This example illustrates the importance of identifying virus isolates. Both A. segetum (P) NPV and A. segetum (F) NPV were isolated from the same host species but are distinct viruses. Previous studies have failed to distinguish between these isolates (Lipa et al., 1971; Burgerjon et al., 1975). It is interesting to note that while Vlak et al. (1981) found considerable similarity between the EcoRI profile of M. brassicae NPV DNA and H. armigera mNPV DNA, the M. brassicae NPV DNA profile shown in Figure 3.3 is not similar to the H. armigera mNPV DNA profile published by Smith and Summers (1978). It is presumed that here again two separate viruses have been isolated from the same host (H. armigera).

The results of the indirect ELISA tests on antigenic relationships between the baculoviruses confirmed the accepted baculovirus subgroupings. Antisera to the mNPV's reacted most strongly with the homologous virus in most cases, and cross-reacted most strongly with the heterologous mNPV's. Reactions with the GV's were much less intense while the sNPV exhibited variable, sometimes intermediate, reactions. The A. segetum GV capsule antiserum reacted most strongly with the GV's, moderately with mNPV's and weakly with the sNPV. Similar results were found by Rohrman et al. (1981) who found that GV and mNPV I.B. polypeptides were distinct both serologically and in terms of amino acid sequence.

As a result of the analyses presented in this Chapter, it was decided to use electrophoretic separations of genome fragments (generated by restriction enzymes), or genome segments, for identification of progeny viruses in cross transmission experiments (Chapter 5).

CHAPTER 4 INCLUSION BODY STRUCTURE

<u>CONTENTS</u>	<u>PAGE</u>
(1) INCLUSION BODY MORPHOLOGY	133
(2) INTERNAL STRUCTURE OF INCLUSION BODIES	137
(3) CALCULATION OF INCLUSION BODY VOLUME	150
(4) CALCULATION OF NUMBERS OF NUCLEOCAPSIDS AND VIRUS PARTICLES WITHIN INCLUSION BODIES	155
a) NUMBER OF NUCLEOCAPSIDS PER VIRUS PARTICLE	156
b) NUMBER OF NUCLEOCAPSIDS AND VIRUS PARTICLES PER I.B.	158
(5) DISCUSSION	161

CHAPTER 4

INCLUSION BODY STRUCTURE

The structures of the viral I.B.'s were analysed so that figures for oral infectivity (Chapter 6) could be converted into equivalent numbers of virus particles and nucleocapsids. This analysis also allowed comparison of the different ways in which virus particles were 'packaged' in I.B.'s of the various isolates.

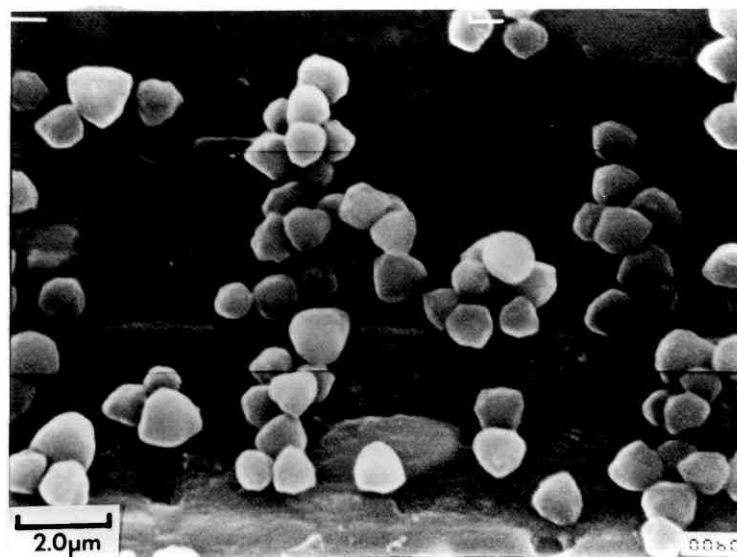
(1) Inclusion Body Morphology

Figure 4.1 shows scanning electron micrographs of A. segetum GV, A. segetum (E) NPV, M. brassicae NPV, P. gamma sNPV and P. gamma CPV I.B.'s. The GV I.B.'s varied little in size or shape, except for rare aberrant forms some of which are arrowed in Figure 4.1. NPV I.B.'s exhibited considerable size variations both between and within isolates. Values for the mean diameters and volumes of polyhedral I.B.'s are given in Section 3.

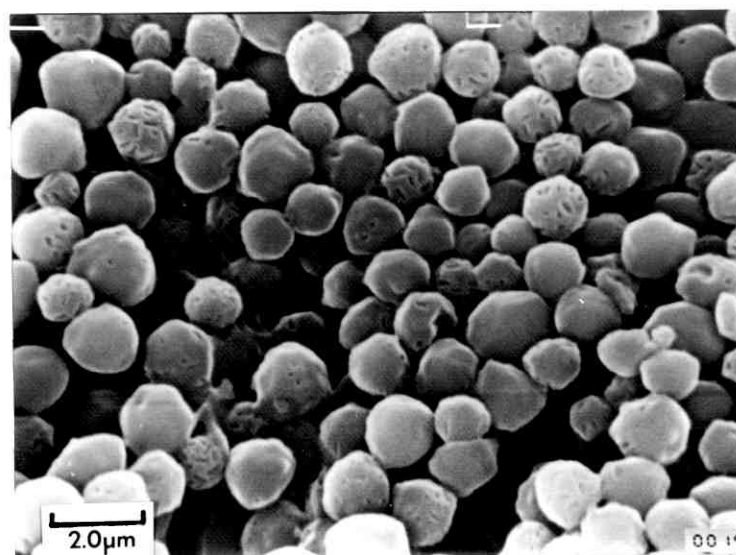
The smallest NPV I.B.'s were those of P. gamma sNPV. These most commonly exhibited rounded tetrahedral symmetry (Figure 4.1). M. brassicae NPV, A. segetum (E) NPV and P. gamma CPV I.B.'s were polyhedral and, in the case of the NPV's, almost spherical in shape. A. segetum (E) NPV I.B.'s, which were the largest of those measured (Section 3) were often fused in pairs. These are shown by phase contrast microscopy in Figure 4.2 and by thin section in Figure 4.9.

The main surface feature revealed by scanning electron microscopy was the surface indentation of M. brassicae NPV I.B.'s. This phenomenon

A



B



C

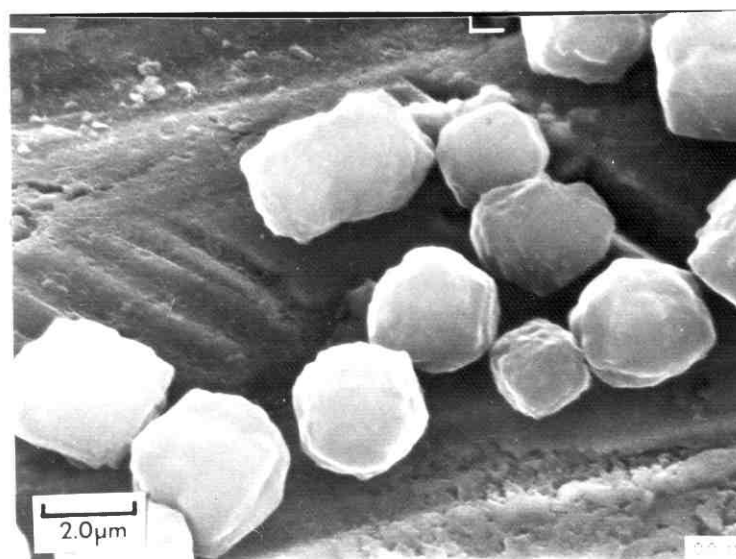


Figure 4.1. Scanning electron microscopy of viral inclusion bodies.
(continued on next page.)

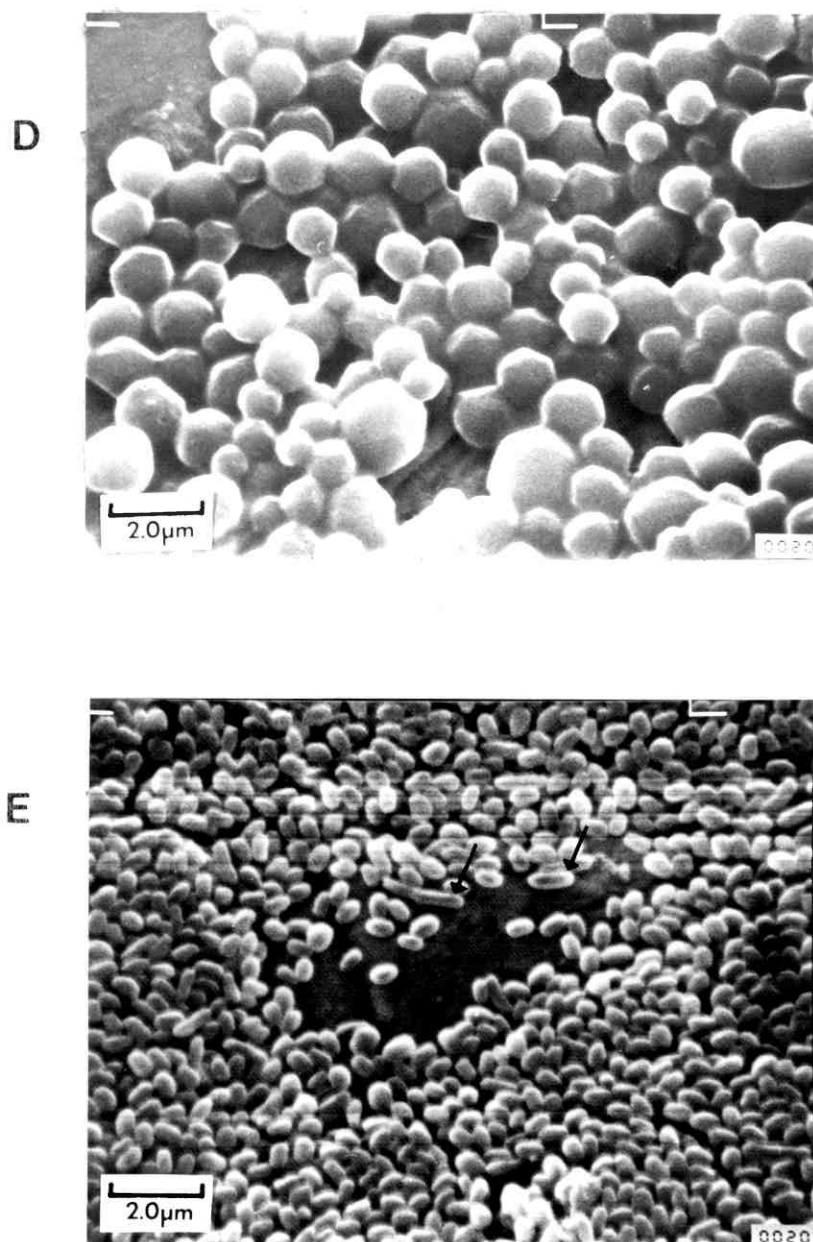


Figure 4.1. (Continued) Scanning electron microscopy of viral inclusion bodies.

A: *P. gamma* sNPV, B: *M. brassicae* NPV,
 C: *A. segetum* (E) NPV, D: *P. gamma* CPV
 E: *A. segetum* GV (arrows indicate aberrant IB's).

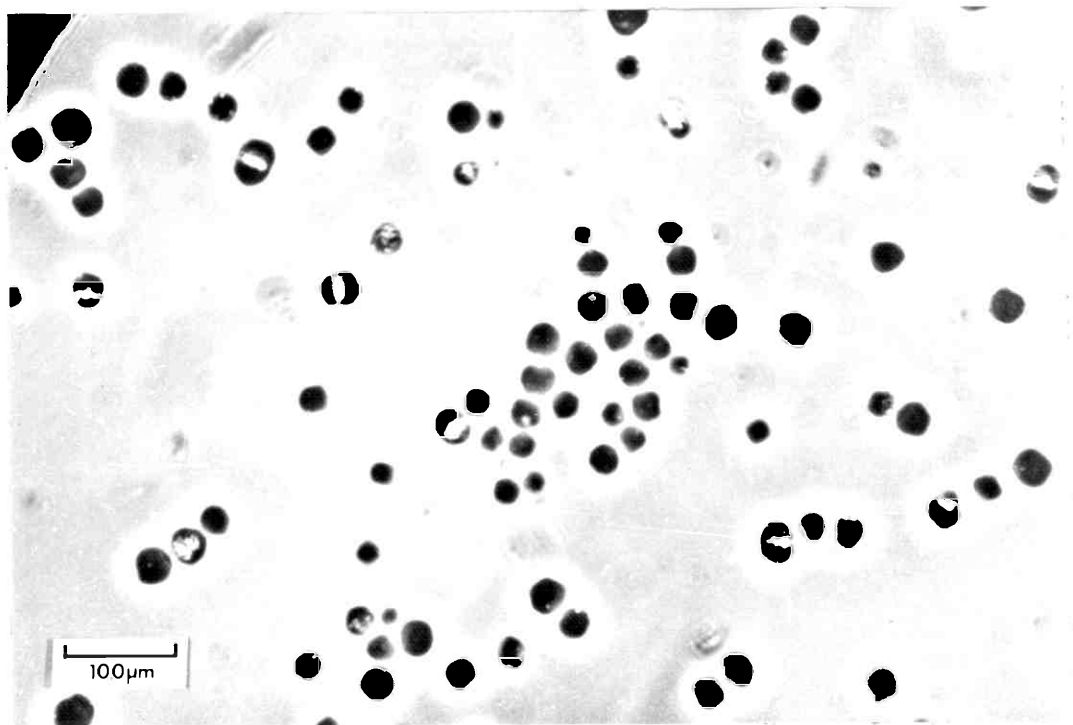


Figure 4.2. Phase contrast microscopy of *A. segetum* (E) NPV, showing some fused IB's.

has been noted by Jurkovicova (1979(b)) who studied the closely related M. brassicae NPV isolate from Holland (Vlak and Groner, 1980). The significance of these indentations is unclear. They may represent sites for virus particle occlusion which have not been filled due to termination of I.B. development. Alternatively there may have been some degradation of the I.B.'s, and loss of virus particles, during I.B. purification.

No pits were found on the surface of P. gamma CPV I.B.'s. These have been found by other workers on CPV I.B.'s (Jurkovicova, 1978(a); Mertens, 1979), and their absence in the present study may be due to the limiting resolution of the microscope.

(2) Internal Structure of Inclusion Bodies

Thin sections of A. segetum GV, P. brassicae GV, P. gamma sNPV and mNPV, M. brassicae NPV, A. segetum (E) NPV, A. segetum (P) NPV and P. gamma CPV I.B.'s are shown in Figures 4.3 to 4.10. The baculovirus particles were typically bacilliform, containing one or more nucleocapsids surrounded by a two-layered membrane or envelope. Measurement of baculovirus nucleocapsids and virus particles showed no systematic difference between the virus isolates except that the diameters of multiply enveloped virus particles were variable and greater than those of singly enveloped virus particles. Measurements were made of 10 virus particles from each sectioned isolate. Only virus particles which possessed clear double membrane structure at both ends, and had therefore been sectioned longitudinally, were used for length measurements. Nucleocapsids measured 250-300 nm x 30-35 nm, while singly enveloped particles measured 300-350 nm x 50-70 nm. Multiply enveloped virus particles up to 240 nm in diameter were observed in A. segetum (E) NPV. Alkali-released virus particles from M. brassicae NPV and A. segetum (E) NPV had similar dimensions to occluded virus particles (Figure 4.11). These figures are in agreement with

those found by other workers (Harrap and Payne, 1979). Virus particles of P. gamma CPV have been previously examined by Martignoni (pers. comm.) who found diameters of 60-70 nm. In most I.B. sections the crystalline lattice of the matrix protein was evident. The distance between protein units was 4-5 nm for baculoviruses and CPV I.B.'s. This is similar to the figure of 4 nm given by Harrap (1972(a)) for the matrix of Lymantria (Porthetria) dispar NPV and Aglais urticae NPV I.B.'s. NPV I.B.'s exhibited a darker staining border in thin section. On alkaline dissolution the matrix lattice breaks down releasing virus particles and a bag-like membrane (Figure 4.11) which may correspond to the border seen in thin section. Partial dissolution can leave the virus particles trapped within this membrane (Figure 4.12). The presence of such a membrane has been reported previously for NPV I.B.'s (Harrap, 1972(a); Jurkovicova, 1979(b)) but not for CPV I.B.'s (Payne, 1981(b)). A. segetum (P) NPV I.B.'s were unusual in often having several membrane layers external to and separate from the polyhedral membrane (Figure 4.8). The significance of these structures was unclear, but it is unlikely that they were artifacts as they have been independently observed by Lipa et al. (1971) in A. segetum (P) NPV.

The majority of GV I.B.'s contained one singly enveloped virus particle (Figures 4.3, 4.5). Very occasionally, virus particles were found containing two nucleocapsids in the case of A. segetum GV but not P. brassicae GV (Figures 4.4, 4.5). A. segetum (E) NPV, A. segetum (P) NPV, M. brassicae NPV and P. gamma mNPV I.B.'s contained singly and multiply enveloped virus particles (Figures 4.7-4.9). These could be resolved into separate bands by isopycnic sucrose gradient centrifugation of alkali dissolved I.B.'s (e.g. Figure 4.13). A. segetum (F) NPV also produced multiple bands by this technique, while P. gamma sNPV and the GV's

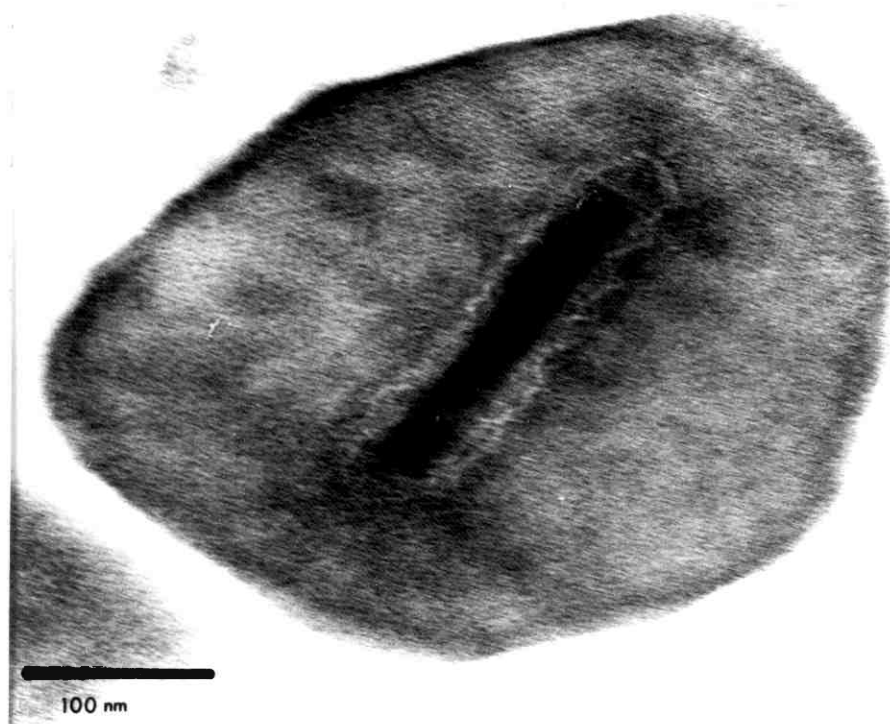
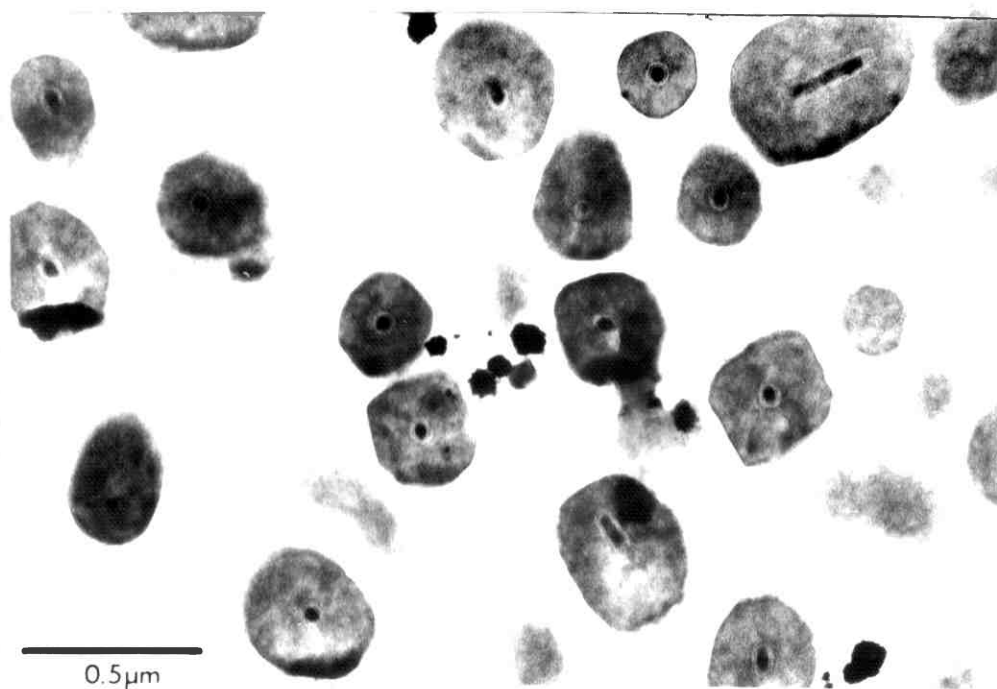
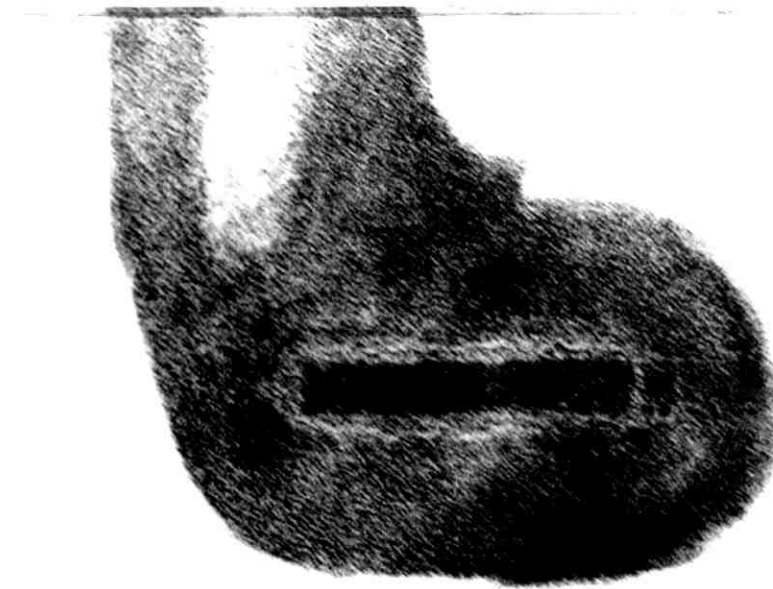
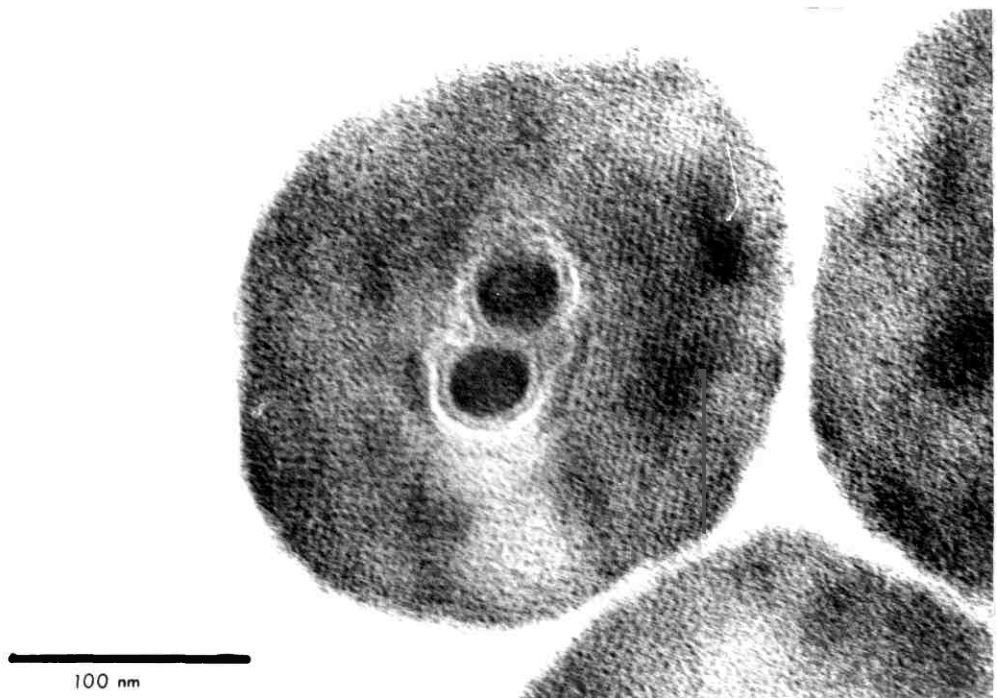


Figure 4.3. Thin sections of *A. segetum* GV IB's.



100 nm



100 nm

Figure 4.4. Thin section of *A. segetum* GV aberrant IB's.

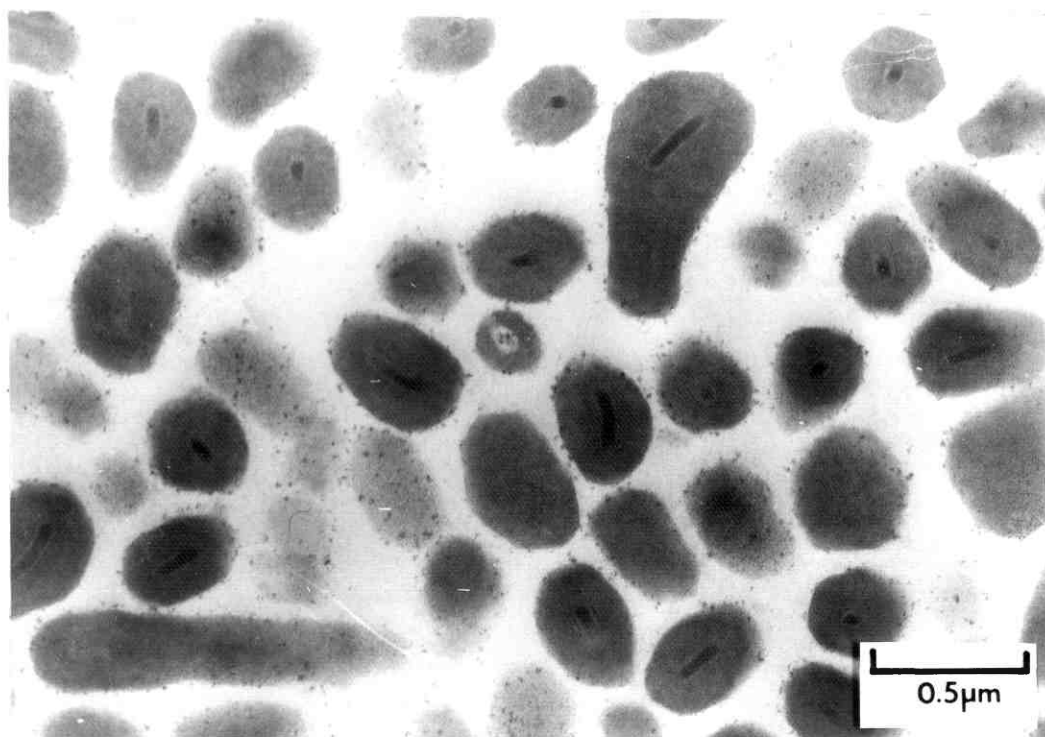
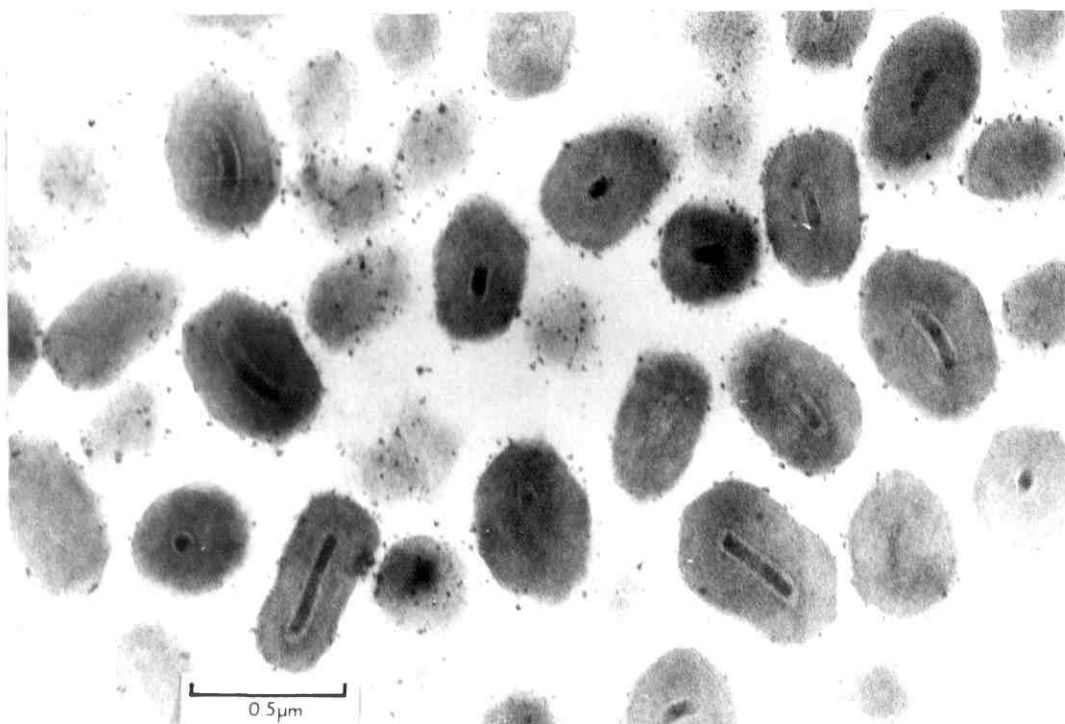


Figure 4.5. Thin sections of *P. brassicae* GV IB's, showing some aberrant forms in the lower photograph.

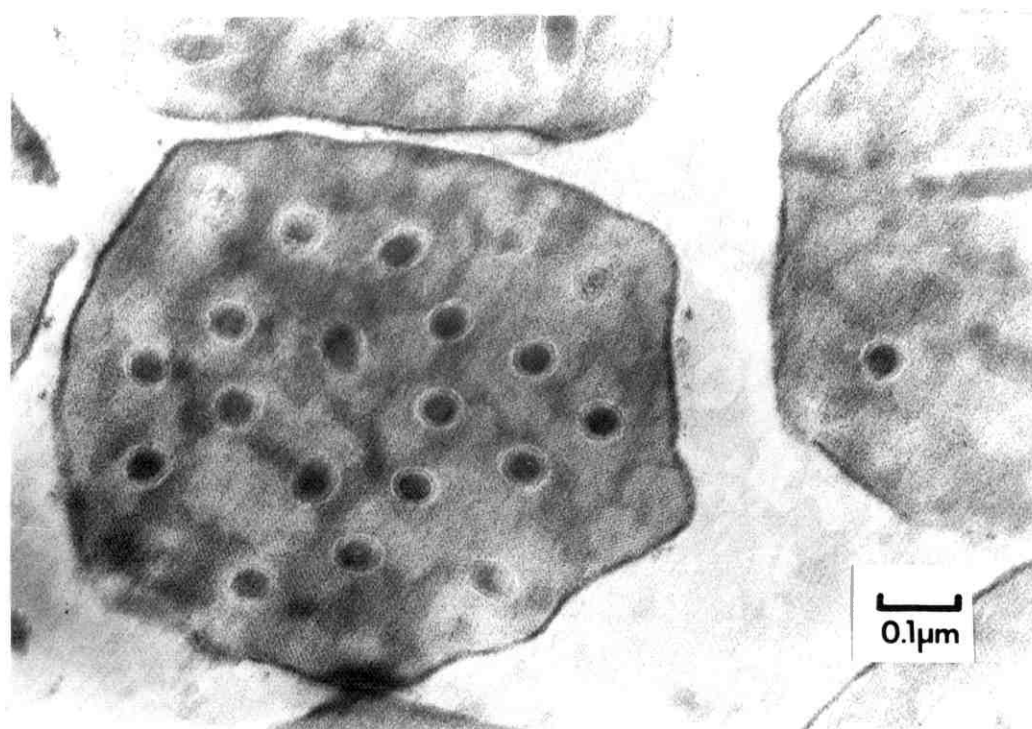
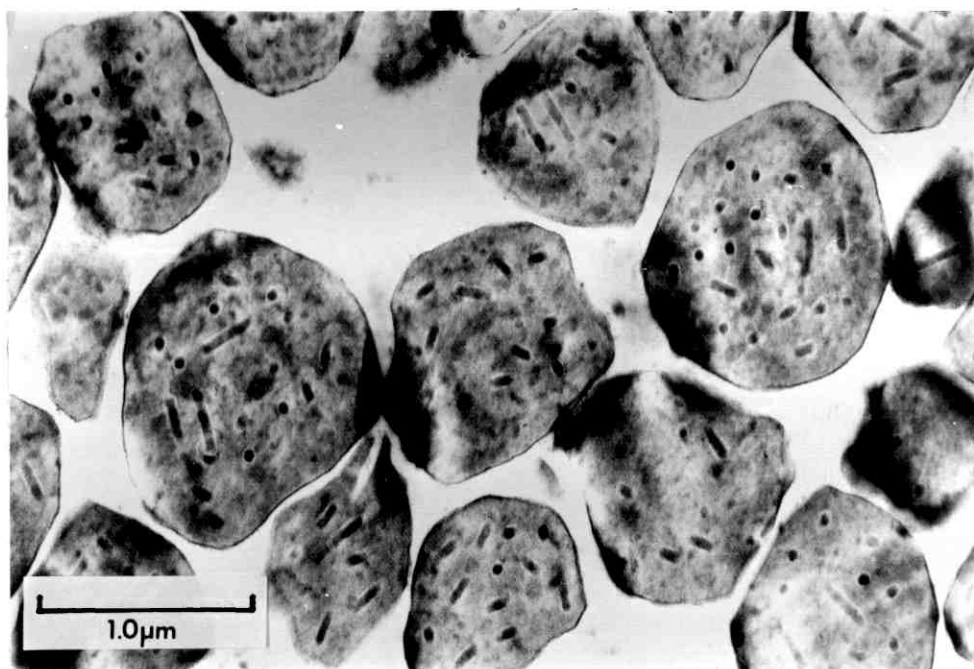


Figure 4.6. Thin sections of *P. gamma* sNPV IB's.

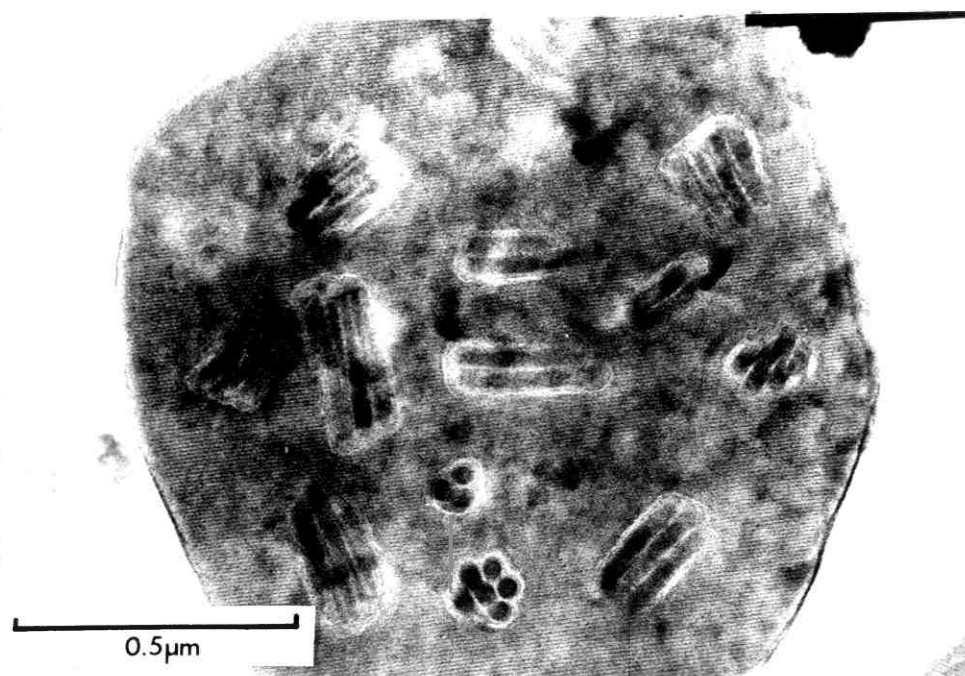
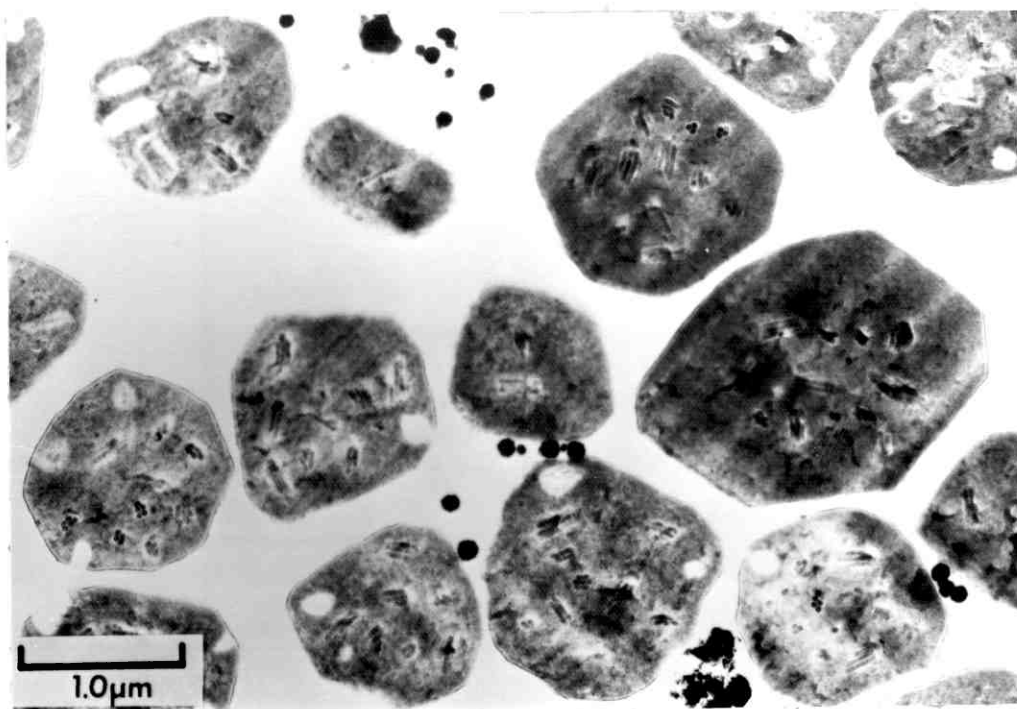


Figure 4.7. Thin sections of *P. gamma* mNPV IB's.

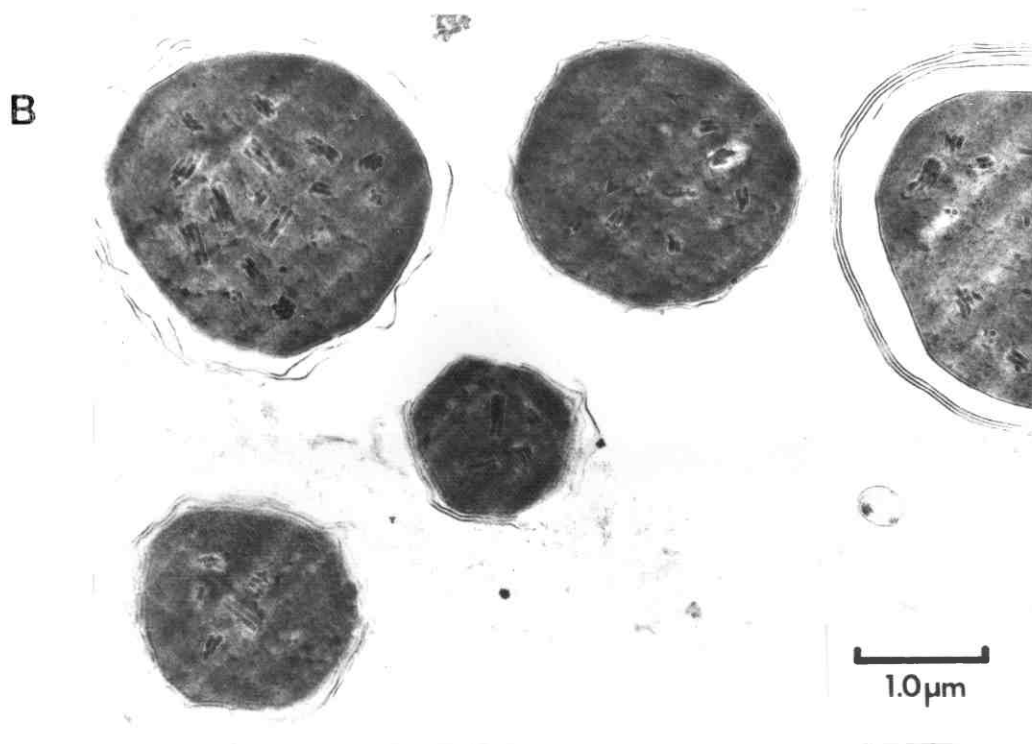
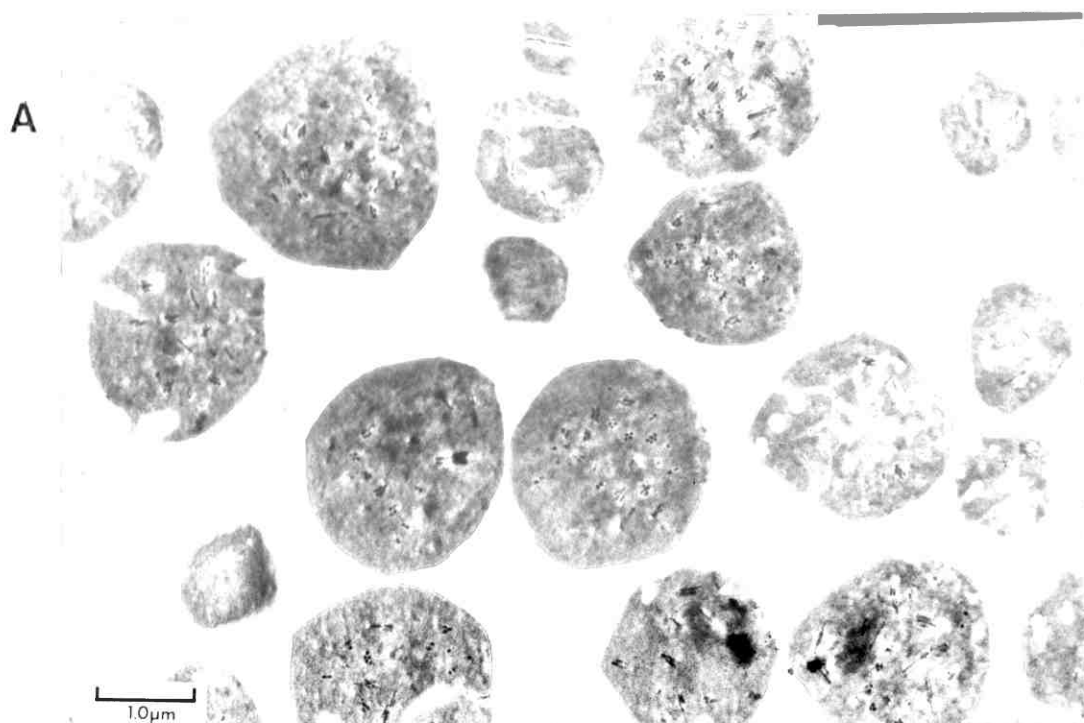


Figure 4.8. Thin sections of *M. brassicae* NPV IB's (A) and *A. segetum* (P) NPV IB's (B).

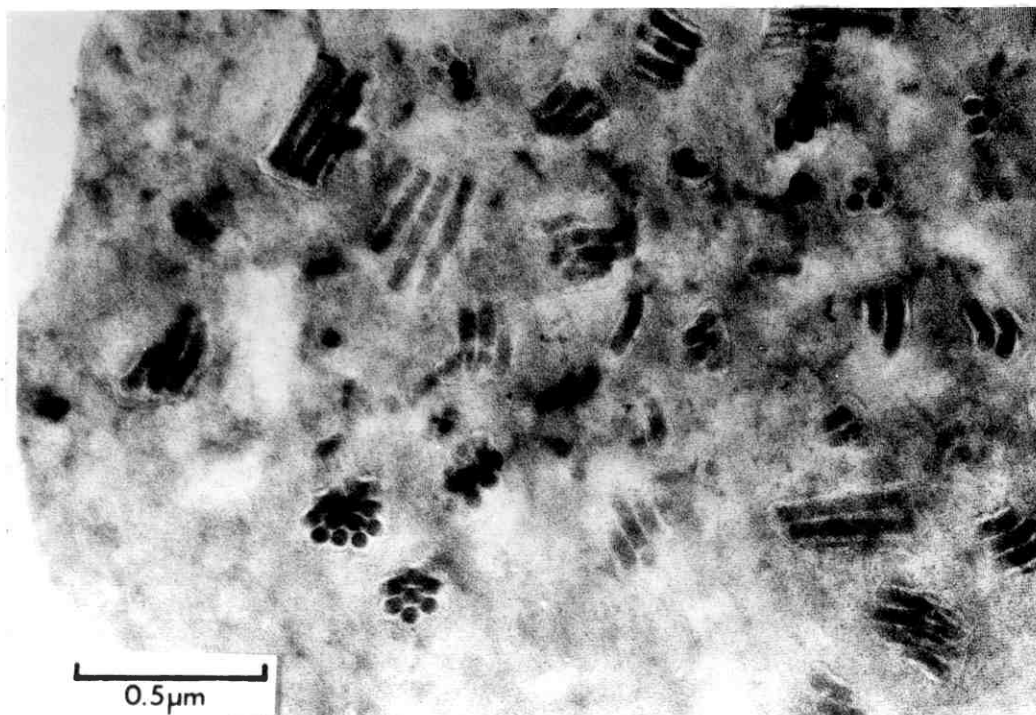
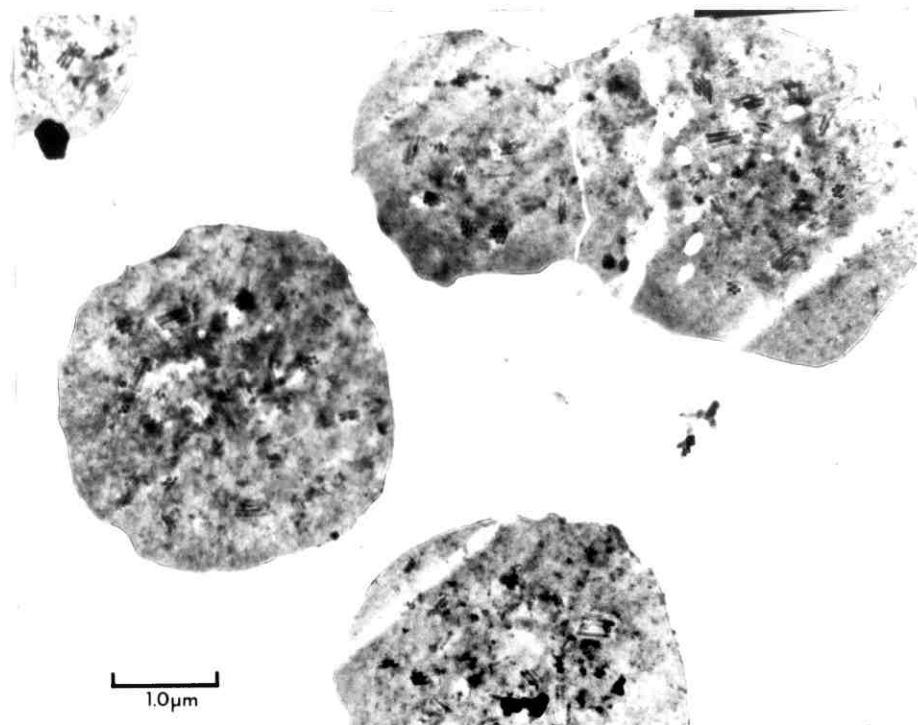


Figure 4.9. Thin sections of *A. segetum* (E) NPV IB's.

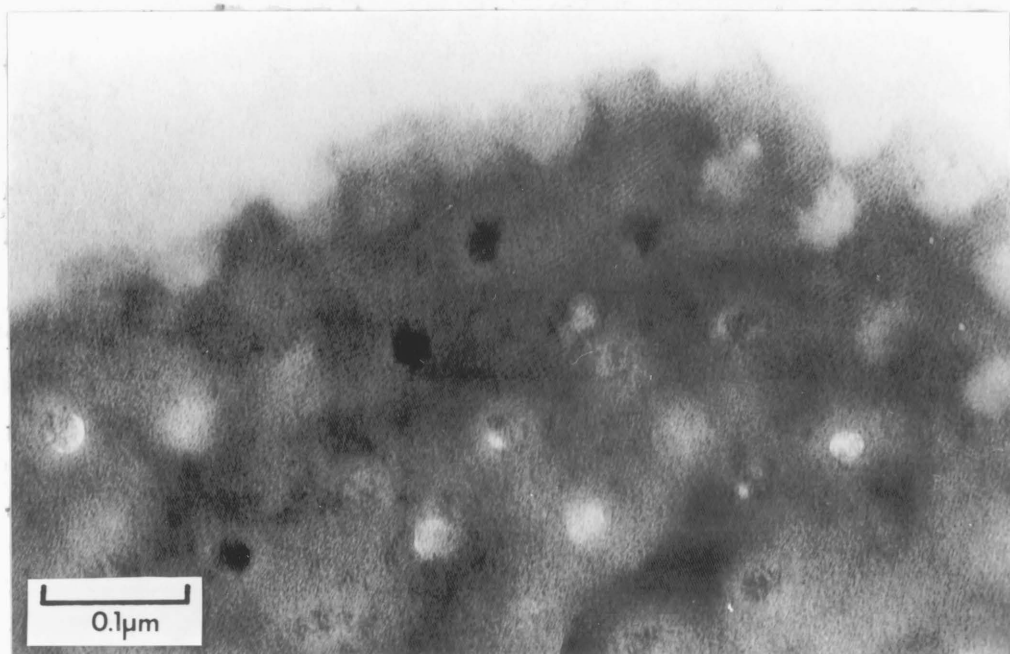
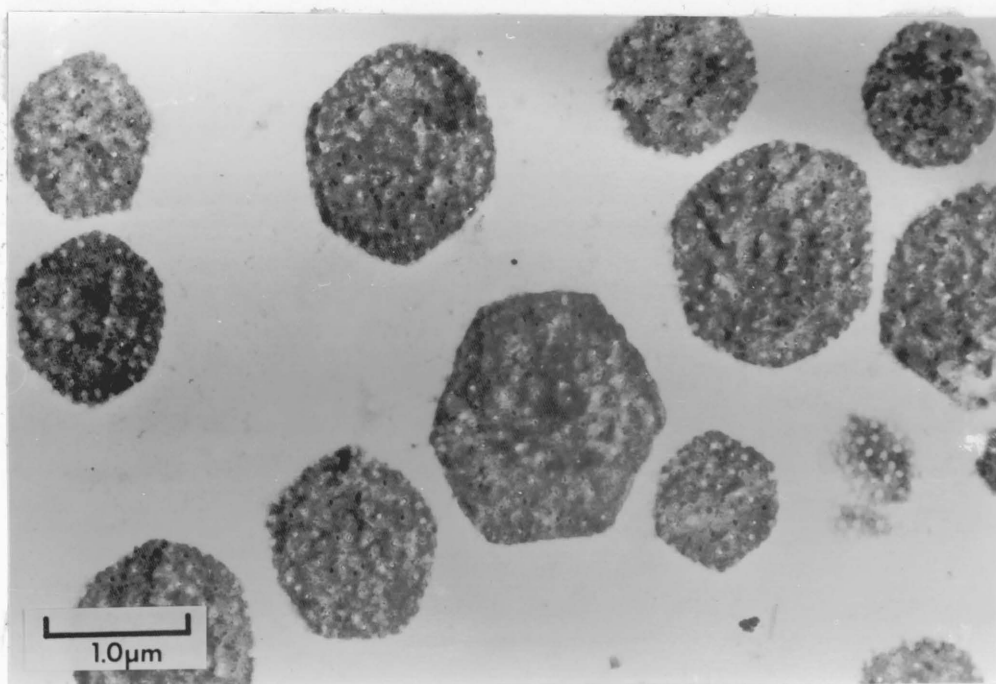
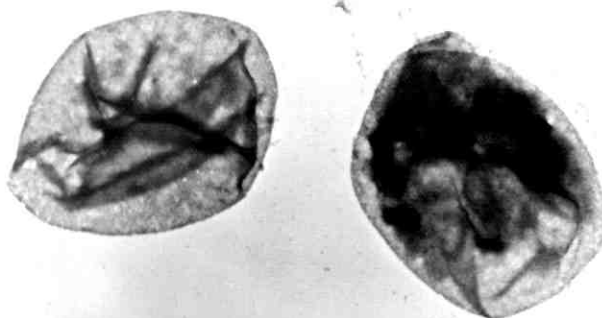


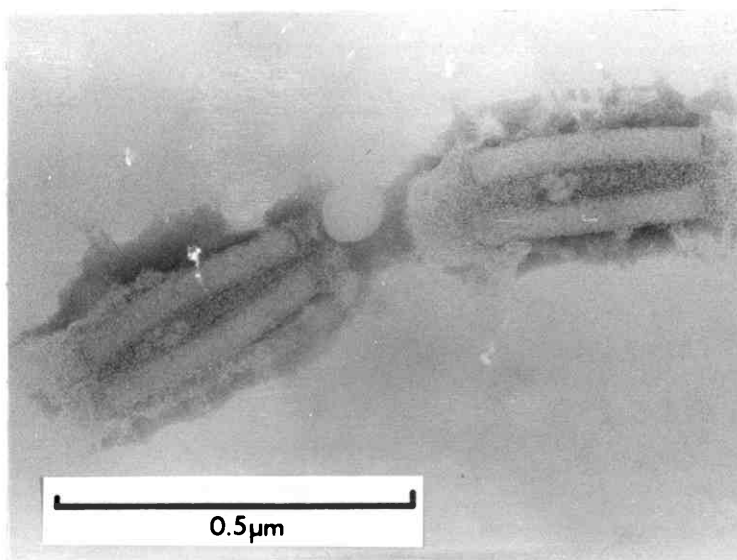
Figure 4.10. Thin sections of *P. gamma* CPV IB's.

A



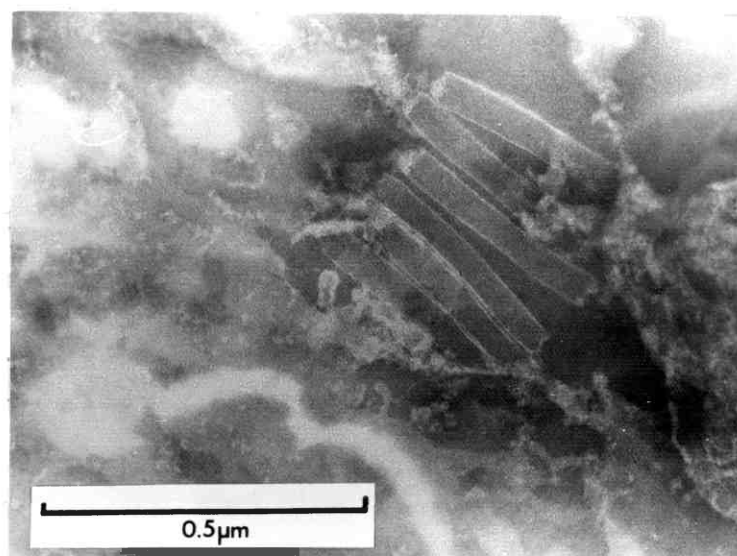
1.0 μm

B



0.5 μm

C



0.5 μm

Figure 4.11. Negative stained preparations from alkaline dissolved NPV IB's. A: *Autographa californica* NPV polyhedral membrane. B,C: *A. segetum* (E) NPV virus particles.

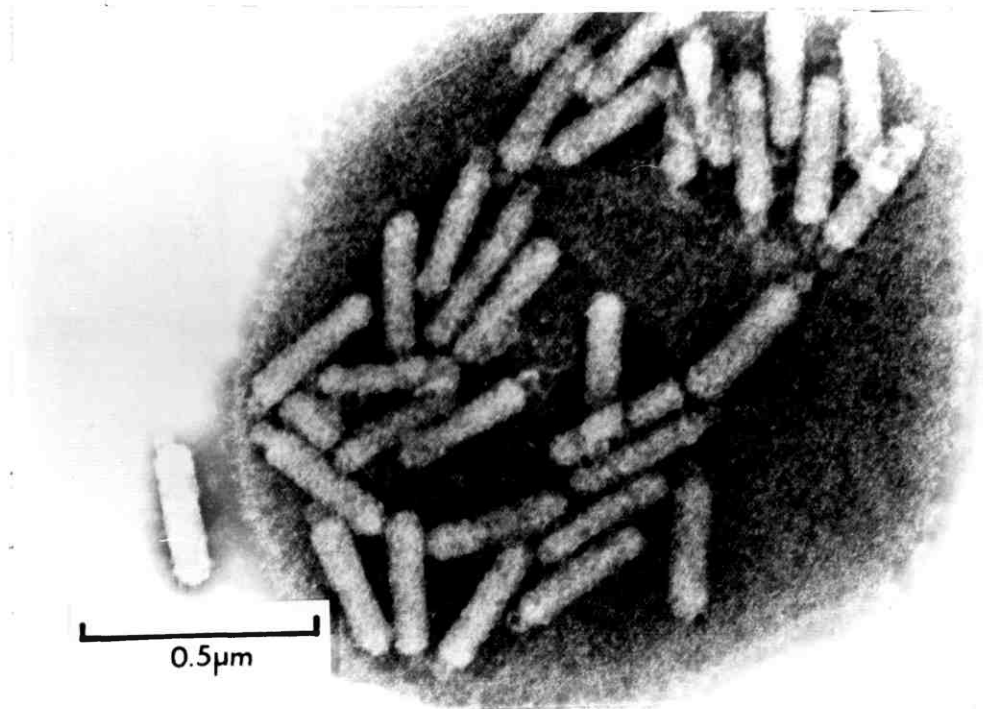
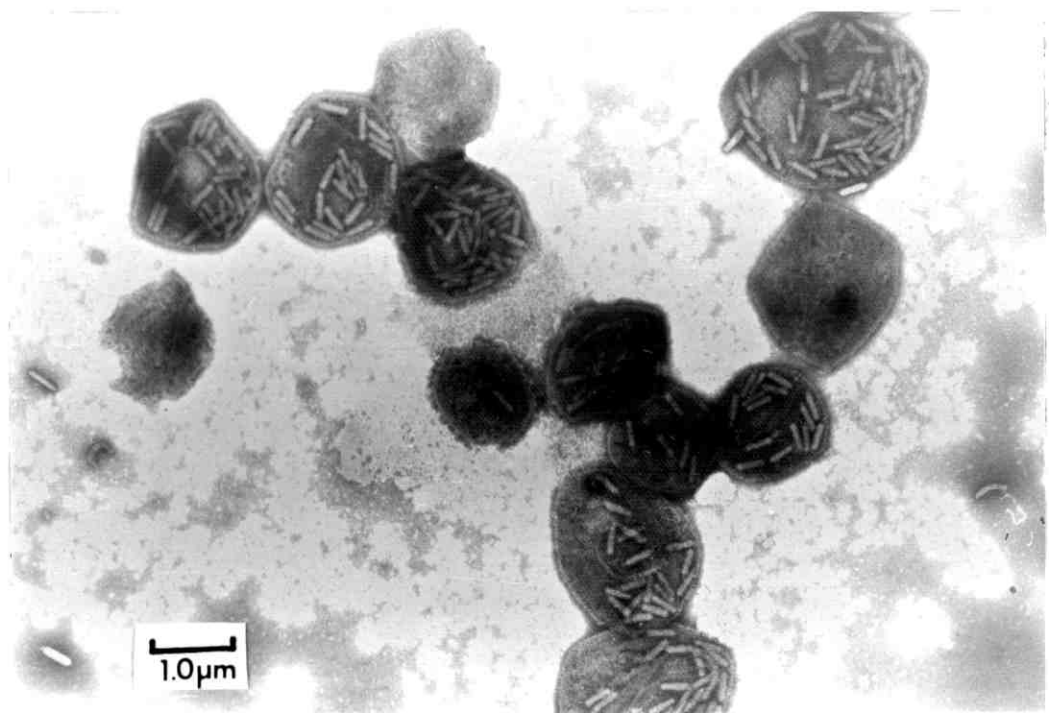


Figure 4.12. Negative stained preparations of *P. gamma* sNPV, partly dissolved IB's.

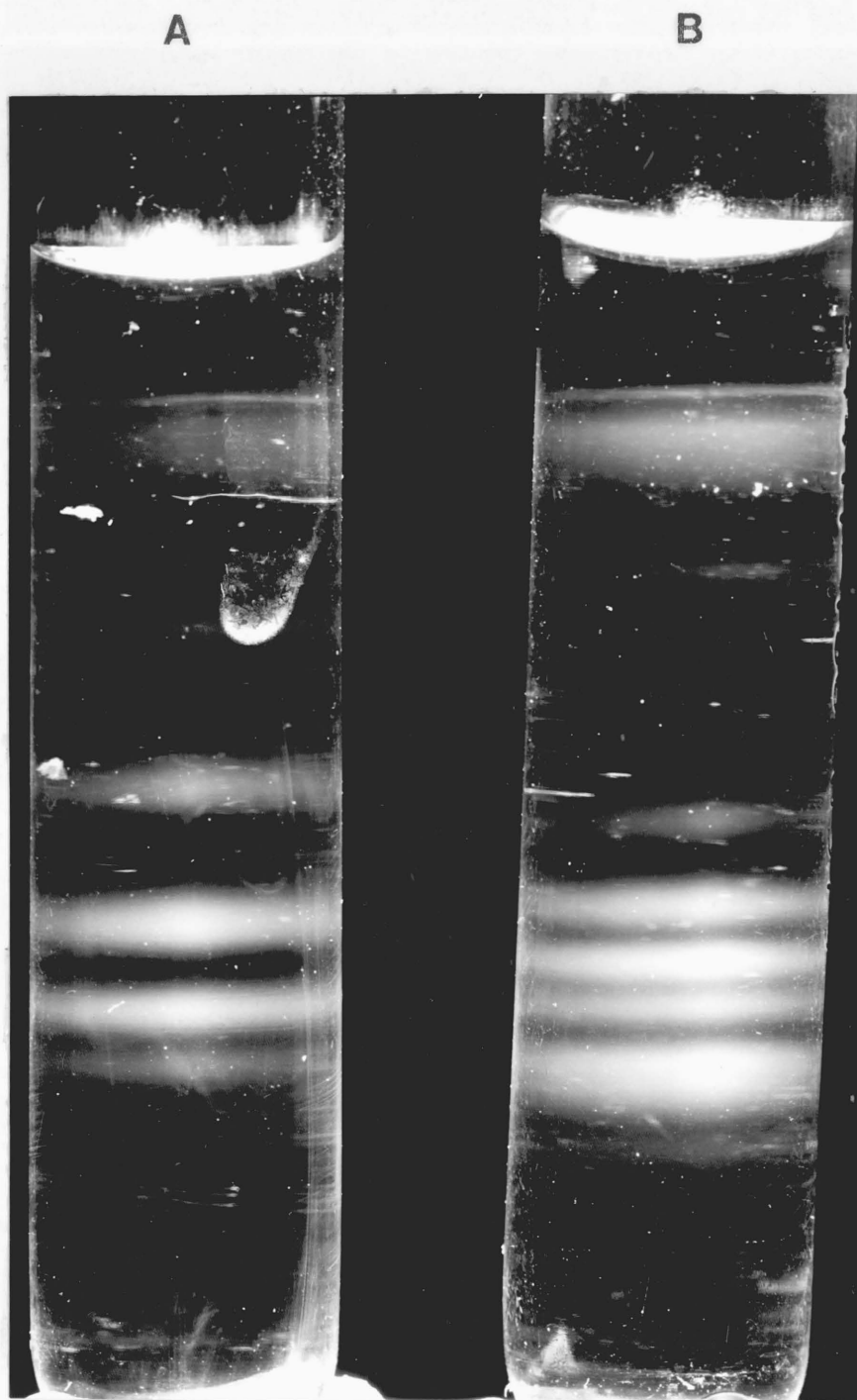


Figure 4.13. Isopycnic sucrose gradient centrifugation of dissolved *M. brassicae* NPV (A) and *A. segetum* (E) NPV (B). 45 - 65% Sucrose in tris/Hcl 0.05M, pH 7.8.

produced only one band (not shown).

P. gamma sNPV I.B.'s contained only singly enveloped virus particles when examined by thin sectioning (Figure 4.6) or by negative staining of partly dissolved I.B.'s (Figure 4.12). This isolate was, therefore, clearly distinct from an mNPV isolated from P. gamma (Chapter 3) which contained multiply enveloped virus particles in thin sections (Figure 4.7). It is interesting that P. gamma sNPV had a higher density of virus particles per I.B. unit section area than the mNPV's (Section 4).

In parallel with the scanning electron micrographs, thin sections of M. brassicae NPV I.B.'s showed surface indentations, as well as internal cavities and cracks (Figure 4.8). Brown and Kelly (1980) also noted this phenomenon when studying the same isolate. It was rarely found in the other NPV's studied. P. gamma CPV I.B.'s exhibited small holes of 30-50 nm, particularly on the surface, which may be the result of virus particle loss (Figure 4.10).

(3) Calculation of I.B. Volume

The mean volumes of NPV and CPV I.B.'s were calculated to estimate the mean number of nucleocapsids and virus particles within the I.B.'s. This estimation also requires calculation of the number of nucleocapsids and virus particles per unit volume (Section 4). Polyhedral diameters were measured by phase contrast microscopy (Chapter 2). For the purposes of this measurement the polyhedra were assumed to be spheres. Small errors resulting from this assumption were negligible in terms of the large differences in mean volume found between different isolates (see below).

In most cases 50 polyhedra were measured. In all cases the distribution of diameters were not significantly different from a normal distribution when tested by the Kolmogorov-Smirnov non-parametric test ($p > 0.05$; Meddis, 1975). The mean volume of each isolate was calculated from the mean and variance of its diameter as described in Appendix 2(a).

Table 3 lists the mean diameters and mean volumes of I.B.'s of the six main polyhedral viruses in this study, and two additional preparations of M. brassicae NPV grown in A. segetum and P. gamma larvae. These preparations were identified as M. brassicae NPV by restriction endonuclease analysis (Chapter 5).

Polyhedral volumes had distributions which were not significantly different from the normal distribution when tested by the Kolmogorov-Smirnov test ($p > 0.05$). The mean volumes of the isolates were compared by Student's t-test (Snedecor and Cochran, 1967). For this purpose each baculovirus was only compared with baculoviruses immediately adjacent in order of size. Where there were significant differences in the variance of the volumes of isolates being compared (as shown by an F-test of variance equality, Snedecor and Cochran, 1967, p.117) the number of degrees of freedom was halved (Snedecor and Cochran, 1967, p.114).

The mean volume of A. segetum (E) NPV I.B.'s was significantly greater than that of A. segetum (F) NPV I.B.'s ($p < 0.001$), which in turn was greater than that of A. segetum (P) NPV I.B.'s ($p < 0.001$).

M. brassicae NPV I.B.'s grown in M. brassicae and in A. segetum could not be distinguished on the basis of mean size ($p > 0.05$). However, M. brassicae NPV I.B.'s grown in P. gamma were significantly smaller than the other two preparations ($p < 0.005$). P. gamma sNPV I.B.'s were significantly smaller than those of the smallest of the mNPV samples

Table 3 Polyhedral Size Measurements

	Mean Diameter (μm)	Standard Deviation of Diameter (μm)	Mean Volume (μm^3)	95% Confidence Limits
<u>A. segetum</u> (E) NPV	2.57	0.41	9.71	$\pm$ 1.21
<u>A. segetum</u> (F) NPV	2.10	0.45	5.54	$\pm$ 0.88
<u>A. segetum</u> (P) NPV	1.72	0.24	2.82	$\pm$ 0.32
<u>M. brassicae</u> NPV				
grown in: (i) <u>M. brassicae</u>	1.66	0.27	2.60	$\pm$ 0.34
(ii) <u>A. segetum</u>	1.62	0.27	2.43	$\pm$ 0.32
(iii) <u>P. gamma</u>	1.43	0.31	1.80	$\pm$ 0.28
<u>P. gamma</u> sNPV	1.05	0.11	0.65	$\pm$ 0.07
<u>P. gamma</u> CPV	1.52	0.30	2.05	$\pm$ 0.30

(M. brassicae NPV grown in *P. gamma*) ($p < 0.001$).

Previous studies of I.B. diameter have been carried out for two of the isolates in Table 3, although only the ranges of diameter were given. Vlcek and Groner (1980) found that the diameter of M. brassicae NPV ranged from 1 to 2.6 μm , while Lipa et al. (1971) measured A. segetum (P) NPV I.B.'s ranging in diameter from 1 to 4.3 μm . These are similar to the values found in the present study. More convincing evidence for the validity of the mean polyhedral volumes given in Table 3 is shown in Figure 4.14. Here the mean polyhedral volume for 6 of the NPV preparations was plotted against the mean protein content per I.B. for that preparation. The latter value was calculated as the total protein content per unit volume of I.B. suspension divided by the concentration of I.B.'s per unit volume. A very good fit was obtained with the regression line shown (correlation coefficient, $r = 0.99$; $p < 0.001$; Meddis, 1975).

It seems that different batches of one virus may have different mean I.B. volumes. This is probably due to variation in physiological conditions in the batches of larvae used, rather than to selection of certain I.B. size fractions during purification. Attempts were made to artificially select for small A. segetum (E) NPV I.B.'s by removing the upper fraction (approximately 10%) of I.B.'s from a 30-90% (v/v) glycerol gradient after centrifugation for 10 minutes at 3.5×10^3 r.p.m. Although this fraction exhibited a mean I.B. diameter around 10% lower than the starting preparation of I.B.'s, this is less than the difference between A. segetum (F) NPV and A. segetum (E) NPV and it is unlikely that normal purification procedures would result in selection of such a biased I.B. sample.

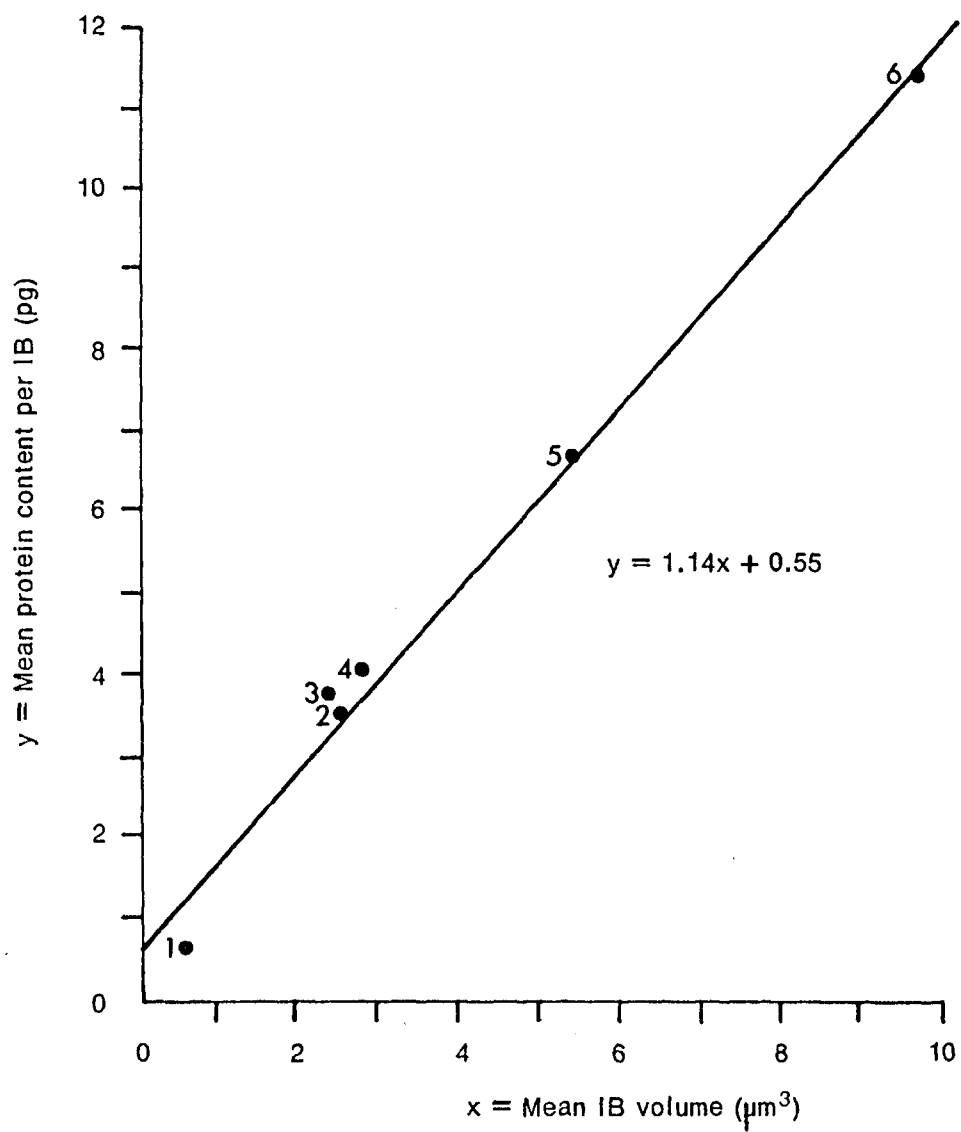


Figure 4.14. Relationship between IB volume and protein content for six NPV preparations.

Key: 1, *P. gamma* sNPV; 2, *M. brassicae* NPV grown in *M. brassicae*; 3, *M. brassicae* NPV grown in *A. segetum*; 4, *A. segetum* (P) NPV; 5, *A. segetum* (F) NPV; 6, *A. segetum* (E) NPV

The most notable feature of mean I.B. volumes shown in Table 3 is the small range ($1.8-2.8 \mu\text{m}^3$) shown by the M. brassicae NPV preparations, A. segetum (P) NPV and P. gamma CPV. The A. segetum (E) NPV and A. segetum (F) NPV I.B.'s were considerably larger than this group, while P. gamma sNPV I.B.'s were considerably smaller.

The I.B.'s of A. segetum GV and P. brassicae GV were measured using thin sections. Length measurements were taken from I.B.'s where the virus particle was in longitudinal section, and represented an average value taken over the curved ends of I.B.'s. The results were the mean of 5-10 measurements in each case:

	Diameter (μm)	Length (μm)	Volume (μm^3)*
<u>A. segetum</u> GV	0.33	0.45	0.038
<u>P. brassicae</u> GV	0.26	0.41	0.022

* calculated as $(\text{radius})^2 \times \text{length} \times \pi$

Lipa and Ziemnicka (1971) described a GV of A. segetum with I.B. length $0.40-0.50 \mu\text{m}$ and Brown et al. (1977) estimated the dimensions of P. brassicae GV I.B.'s as $0.35 \times 0.20 \mu\text{m}$. These figures are in reasonable agreement with the values found in the present study. The protein contents per I.B. of A. segetum GV and P. brassicae GV were 0.045 pg and 0.029 pg respectively, confirming that A. segetum GV I.B.'s are the larger. The protein contents per unit I.B. volume were $1.18 \text{ pg}/\mu\text{m}^3$ for A. segetum GV and $1.32 \text{ pg}/\mu\text{m}^3$ for P. brassicae GV, results similar to those for the NPV's (Figure 4.14).

(4) Calculation of Numbers of Nucleocapsids and Virus Particles Within Inclusion Bodies

This study was done with M. brassicae NPV, A. segetum (E) NPV, A. segetum (P) NPV, P. gamma sNPV and P. gamma CPV. The mean number of

nucleocapsids and virus particles per GV I.B. was very close to one, the proportion of aberrant I.B.'s being very low (Section 2).

a) Number of nucleocapsids per virus particle

A. segetum (E) NPV, A. segetum (P) NPV and M. brassicae NPV I.B.'s were examined by thin section to determine the number of virus particles in each size class. Only virus particles which had been sectioned transversely were counted. Approximately 100 virus particles in total were counted for each virus. The results are shown in Table 4. In the case of A. segetum (E) NPV and M. brassicae NPV but not A. segetum (P) NPV, the distribution of virus particles among the different size classes was significantly different from a normal distribution when tested by the Kolmogorov-Smirnov test ($p < 0.05$). The large sample size permitted comparison of the means by Student's t-test (Snedecor and Cochran, 1967, p.51). While A. segetum (E) NPV and A. segetum (P) NPV were not significantly different by this test ($p > 0.05$), M. brassicae NPV had a significantly lower mean number of nucleocapsids per virus particle than A. segetum (P) NPV ($p < 0.001$). Although the two A. segetum NPV isolates had a similar mean number of nucleocapsids per virus particle, the distributions of virus particles among the size classes were different.

In the case of A. segetum (E) NPV and M. brassicae NPV, the different distributions of virus particles within the size groups was confirmed by the pattern of virus particle bands found on isopycnic sucrose gradients of dissolved polyhedra (Figure 4.13). When A. segetum (E) NPV and A. segetum (F) NPV dissolved I.B.'s were run on parallel sucrose gradients, the virus particle bands showed identical patterns (not shown). This suggests that the distributions of virus particle size classes within I.B.'s of these two isolates are identical. Care must be taken, however, when analysing band patterns on density gradients. The intensity of bands depends on the size and concentration of virus particles within the bands. Some bands may be composed of mixtures of virus particle size classes or of degraded virus particle

Table 4

Distribution of No. Nucleocapsids Per Virus Particle in
A. segetum (E) NPV, A. segetum (P) NPV and M. brassicae NPV

Nucleocapsids/ Virus Particle	% Virus Particles in Each Size Class		
	<u>A. segetum (E) NPV</u>	<u>A. segetum (P) NPV</u>	<u>M. brassicae NPV</u>
1	33	17	35
2	5	10	21
3	8	16	23
4	13	21	16
5	11	16	5
6	6	2	
7	14	10	1
8	7	5	
9	1		
10		1	
11	2		
12			
13	1		

Number of Nucleocapsids/Virus Particle:

	Mean	95% Confidence Limits
<u>A. segetum (E) NPV</u>	4.04	$\pm$ 0.56
<u>A. segetum (P) NPV</u>	3.89	$\pm$ 0.42
<u>M. brassicae NPV</u>	2.40	$\pm$ 0.26

fragments (Cibulsky et al., 1977; Jurkovicova, 1979(b)).

When Vlak and Gröner (1980) analysed two isolates of M. brassicae NPV they found 1-8 nucleocapsids per virus particle, a similar range to that found in the present study. However, when Lipa et al. (1971) examined the A. segetum (P) NPV isolate they found 4-8 nucleocapsids per virus particle, while in the present study 43% of virus particles had 3 nucleocapsids or fewer. The A. segetum (E) NPV isolate has not been previously studied in this way.

b) Number of virus particles per I.B.

The mean number of virus particles per I.B. was calculated by a novel method, from the number seen in thin sections. To perform this calculation it was necessary to know the mean I.B. volume (Section 3) and the volume of the I.B. which was sampled by the thin section. The latter value was given by the area of the thin section multiplied by the sample thickness which was itself dependent on the dimensions of the virus particles. The calculation is given in Appendix 2(b). The following values were obtained for the sample thickness for each isolate:

<u>A. segetum</u> (E) NPV	323 nm
<u>A. segetum</u> (P) NPV	312 nm
<u>M. brassicae</u> NPV	304 nm
<u>P. gamma</u> sNPV	285 nm
<u>P. gamma</u> CPV	145 nm

The number of virus particles per I.B. section was counted in 25 sections for each virus and converted to mean number per μm^2 for that virus. Sections in which virus particles appeared to be non-randomly orientated were not used. These occurred at the edge of the I.B.'s where the virus particles often laid parallel to the surface. The distribution of numbers

of virus particles per μm^2 was not significantly different from a normal distribution when tested by the Kolmogorov-Smirnov test ($p > 0.05$).

The following results were calculated for the mean number of virus particles per μm^2 . The 95% confidence limits are also shown:

<u>A. segetum</u> (E) NPV	5.6 $\pm$ 0.6
<u>A. segetum</u> (P) NPV	6.1 $\pm$ 0.8
<u>M. brassicae</u> NPV	5.5 $\pm$ 0.8
<u>P. gamma</u> sNPV	17.0 $\pm$ 2.1
<u>P. gamma</u> CPV	37.6 $\pm$ 4.3

The mean numbers of virus particles per μm^3 were calculated by dividing the number per μm^2 by the sample thickness (both figures given above).

The results are given in Table 5. The numbers of virus particles and nucleocapsids per I.B. were calculated by multiplying mean number of virus particles per μm^3 by mean I.B. volume and by mean number of nucleocapsids per virus particle (Appendix 2(c)). These results are also given in Table 5, where figures for the GV's are included for comparison.

Table 5 shows that I.B.'s of the three mNPV's had very similar concentrations of virus particles. These were not significantly different from each other by Student's t-test ($p > 0.05$). P. gamma sNPV I.B.'s had more than three times the virus particle concentration of the mNPV's and the mean was significantly different from that of A. segetum (P) NPV ($p < 0.001$). The GV's showed a similar virus particle:I.B. volume ratio to the NPV's while P. gamma CPV I.B.'s had a much higher virus particle concentration.

The calculated mean number of virus particles per I.B. in P. gamma sNPV was similar in magnitude to the number counted in partly dissolved

Table 5 No. Virus Particles and Nucleocapsids Per Inclusion Body

Virus	No. Virus Particles/ μm^3		No. Virus Particles/IB		No. Nucleocapsids/IB	
	Mean	95% Confidence Limits	Mean	95% Confidence Limits	Mean	95% Confidence Limits
<u>A. segetum</u> (E) NPV	17.3	± 1.9	168.0	± 27.4	673.7	± 144.6
<u>A. segetum</u> (P) NPV	19.6	± 2.6	55.3	± 9.6	215.0	± 44.2
<u>M. brassicae</u> NPV	18.1	± 2.6	47.1	± 8.7	100.3	± 24.1
<u>P. gamma</u> sNPV	59.6	± 7.4	38.7	± 6.2	38.7	± 6.2
<u>A. segetum</u> GV	26.3*	—	1.0	—	1.0	—
<u>P. brassicae</u> GV	45.5*	—	1.0	—	1.0	—
<u>P. gamma</u> CPV	259.3	± 29.7	531.6	± 99.2		

* reciprocal of mean I.B. volume

I.B.'s (Figure 4.12), although the presence of free virus particles in this preparation showed that some I.B.'s had lost some or all of their virus particles. A similar figure of 26.4 for mean number of virus particles per I.B. was found by Gregory et al. (1969) for H. zea sNPV, while Smirnof and Ackermann (1977) noted up to 80 virus particles in I.B.s of Thymelicus lineola sNPV. Vlak and Groner (1980) reported 1.4 virus particles per μm^2 of thin section for M. brassicae NPV I.B.'s compared with 5.5 for the present study using the same isolate. Their estimate appears to be incorrect as it would correspond to around 12 virus particles per I.B. (Table 5), whereas most polyhedral sections included at least this many virus particles (Figure 4.8) so the number per I.B. must have been considerably greater.

(5) Discussion

The mean volume of polyhedral virus I.B.'s varied considerably between preparations of that virus (Table 3). The cause of this phenomenon is unknown, but the physiological state of the insects used for virus production may have been important. Variations in I.B. size within one virus preparation (Chapter 1, Section 3) may be associated with the same mechanism. I.B. volume did not seem to be under rigid viral control, although whether I.B.'s were large, medium or small was a characteristic of a particular virus.

The mean number of nucleocapsids per virus particle and the concentration of virus particles in mNPV's have been shown to remain constant after passage in alternative hosts (Tomkins et al., 1980). The similarity of the virus particle band profiles on sucrose gradients between different preparations of the same virus confirmed that the proportions of the

different virus particle size classes remained constant. These two characteristics therefore, appear to be under viral control.

The baculoviruses exhibited a limited range of numbers of virus particles per unit I.B. volume (Table 5) which may indicate some structural limitations to this feature. Within the range shown, the three mNPV's had very similar virus particle concentrations while P. gamma sNPV had a higher concentration. Due to the differences in mean I.B. volume, A. segetum (F) NPV, M. brassicae NPV and P. gamma sNPV contained similar numbers of virus particles per I.B., while A. segetum (E) NPV contained several times as many. When the varying mean numbers of nucleocapsids per I.B. were considered, substantial differences were found in numbers of nucleocapsids per I.B. between these viruses. P. gamma sNPV had a different packaging strategy from the three mNPV's, with a high concentration of singly enveloped virus particles in small I.B.'s rather than a lower concentration of multiply enveloped virus particles in larger I.B.'s. Heliothis zea sNPV also had small I.B.'s, containing a similar number of virus particles to P. gamma sNPV (Gregory et al., 1969), and Thymelicus lineola sNPV had a high density of virus particles in small I.B.'s (Smirnoff and Ackermann, 1977). These studies suggest that the arrangement found in P. gamma sNPV I.B.'s (above) is also true for other sNPV's.

The NPV's contrasted sharply with the GV's which usually had one singly enveloped particle per I.B.

P. gamma CPV had a very high concentration of virus particles, in the order of 10 fold greater than that of the baculoviruses. This may be related to the smaller virus particles of this virus type.

The relationships between the different arrangements of virus particle packaging, and the biological properties of the viruses are considered in Chapters 6 and 8.

CHAPTER 5 HOST RANGE STUDIES

<u>CONTENTS</u>	<u>PAGE</u>
(1) INFECTION TESTS WITH NEONATE LARVAE	165
(2) IDENTIFICATION OF PROGENY VIRUS IN CROSS-INFECTION TESTS	166
a) BACULOVIRUSES	166
b) CYTOPLASMIC POLYHEDROSIS VIRUSES	173
(3) SUSCEPTIBILITY OF <u>M. BRASSICAE</u> TO THREE EUROPEAN ISOLATES OF <u>A. SEGETUM</u> NPV	176
(4) DISCUSSION	177

CHAPTER 5

HOST RANGE STUDIES

The host ranges of the viruses were examined by oral inoculation of neonate larvae. This was done both as a preliminary to the LD50 and LT50 studies described in Chapter 6, and also to compare the host ranges of viruses with different I.B. structure.

(1) Infection Tests With Neonate Larvae

The host ranges were examined in neonates as these are usually the larval stages most susceptible to virus infection (Chapter 1, Section 6(a)). Where these larvae died of an occluded virus infection, older larvae were infected with the original inoculum to produce progeny virus for identification (Section 2).

Two replicates of 25 neonate larvae were dosed orally, as described in Chapter 2, with 10^4 I.B.'s (for NPV's and the CPV) or 10^6 I.B.'s (for GV's) per larva. In addition, 25 control larvae were dosed with virus-free agar/teepol diluent. The 100 fold difference in I.B. dosage between polyhedral viruses and GV's was adopted to approximately equalise the numbers of virus particles used (Table 5). Larvae were regularly examined for mortality up to three weeks after infection, when any larvae still alive were killed. All larvae were examined for the presence of I.B.'s by phase contrast microscopy, as described in Chapter 2.

The presence of I.B.'s was used as the criterion for positive infection in these tests. If limited infections occurred, with no I.B. development, they would not have been detected. It is probable that such incomplete replication would have little ecological significance,

if no occluded progeny virus were produced and no pathological symptoms appeared. Most previous studies of occluded virus host range in vivo have used this criterion for positive infection.

The results of the cross-infection tests in neonate larvae are given in Table 6. P. gamma CPV caused infections in all tested species including P. rapae as well as the noctuids. M. brassicae NPV had the widest host range of the baculoviruses, causing infections in all tested noctuids. The other baculoviruses did not infect hosts outside the homologous subfamily. Additional tests of M. brassicae susceptibility to three A. segetum NPV isolates are described in Section 3.

(2) Identification of Progeny Virus in Cross-Infection Tests

Several studies have shown that, when insects are inoculated with a particular virus, they may die of an infection caused by a different virus, of unknown origin (Chapter 1, Section 4(f)). To confirm cross-transmission results, therefore, it was necessary to identify the virus progeny. The most precise techniques for identification (Chapter 3) were gel electrophoresis of viral polypeptides, nucleic acid segments (CPV's) or restriction enzyme fragments (baculoviruses). These required milligram quantities of I.B.'s, which were most conveniently produced in late instar larvae. The techniques for growing up virus, purifying viral components and electrophoretic separations, have been described (Chapter 2).

a) Baculoviruses

In early studies, SDS-polyacrylamide gel electrophoresis of virus particle polypeptides was used for the identification of baculovirus cross-infection progeny. However, the intensity of some polypeptide

Table 6 Virus Host Range

Virus	Noctuidae						Pieridae
	<u>Agrotis</u> <u>segetum</u>	<u>Agrotis</u> <u>exclamationis</u>	<u>Noctua</u> <u>pronuba</u>	<u>Mamestra</u> <u>brassicae</u>	<u>Lacanobia</u> <u>oleracea</u>	<u>Plusia</u> <u>gamma</u>	<u>Pieris</u> <u>rapae</u>
<u>M. brassicae</u> NPV	+	+	+	+	+	+	-
<u>A. segetum</u> (E) NPV	+	+	(±)	-	-	-	-
<u>P. gamma</u> sNPV	-	-	-	-	-	+	-
<u>A. segetum</u> GV	+	+	±	-	-	-	-
<u>P. brassicae</u> GV	-	-	-	-	-	-	+
<u>P. gamma</u> CPV	n.t.	±	(±)	(±)	±	+	+

Key: - = No Infection; + = Infection, Progeny Confirmed, Same as Inoculum;

± = Infection, No Progeny Confirmation; (±) = Infection, Progeny Different from Inoculum in Some Cases (See Text)

n.t. = Not Tested.

bands varied considerably between progeny viruses which differed by no other criteria (Figure 5.1). This may have been due to varying activity of endogenous alkaline proteases (Chapter 3). Agarose gel electrophoresis of baculovirus DNA restriction endonuclease fragments proved more suitable for progeny identification, as it produced consistent fragment profiles for specific viruses. EcoRI profiles for most of the baculovirus progeny preparations are shown in Figure 5.2, in parallel with profiles of DNA fragments from the original inocula.

Preparations of M. brassicae NPV grown in each of the five non-homologous noctuid species, had identical restriction endonuclease digest profiles to that of the inoculum (Figure 5.2; the profile of the preparation grown in L. oleracea is not shown). Similarly A. segetum (E) NPV and A. segetum GV progeny viruses grown in A. exclamationis were identical with the inoculum. When N. promuba larvae were inoculated with A. segetum (E) NPV, however, the progeny virus had a distinctive profile on agarose gel electrophoresis of the purified nucleic acid (Figure 5.2). This profile had only 7 bands and resembled part of a CPV ds RNA segment profile. Examination of the I.B.'s by negative staining electron microscopy, confirmed that the progeny virus was a CPV. When the nucleic acid preparation was electrophoresed on a 3% polyacrylamide gel (Figure 5.4) the profile was very similar to that of a CPV isolated from N. promuba which had been earlier identified by Payne and Rivers (1976).

While the progeny virus produced in late instar N. promuba larvae inoculated with A. segetum (E) NPV was predominantly a CPV, there may have been some baculovirus present. The electrophoretic profile of undigested nucleic acid on agarose gels included a high molecular weight band with the same mobility as undigested M. brassicae NPV DNA (Appendix 1).

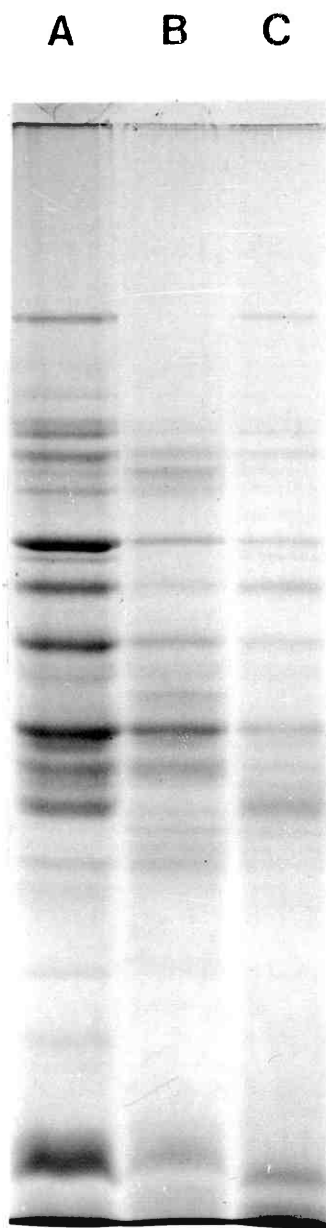


Figure 5.1. Electrophoretic profile of *M. brassicae* NPV virus particle polypeptides, grown in *A. segetum* (A), *M. brassicae* (B), and *A. exclamationis* (C).

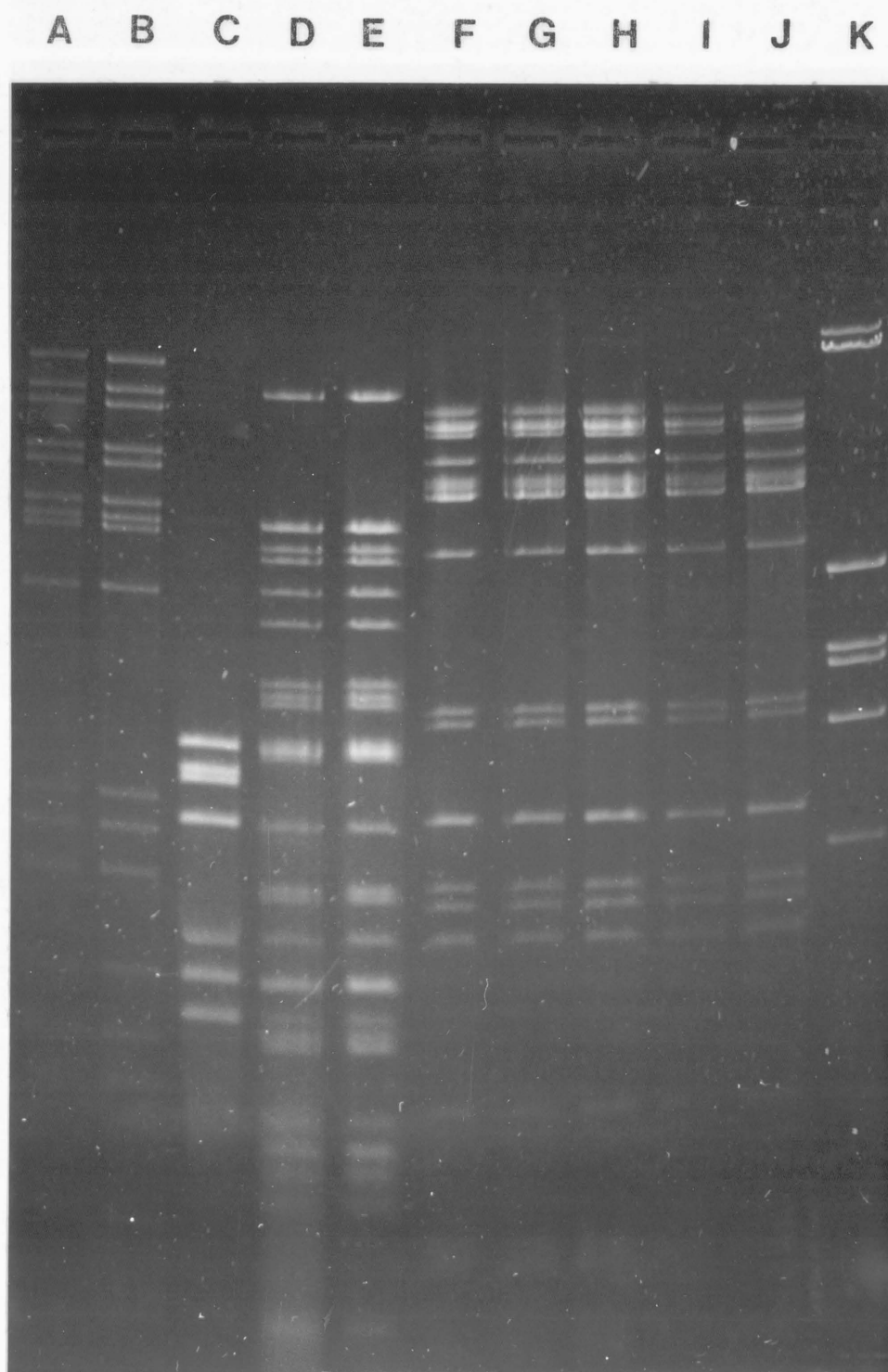


Figure 5.2. Electrophoresis of baculovirus inocula and progeny DNA, EcoRI digests on 1% agarose gel.

Key:

- A: *A. segetum* GV grown in *A. exclamationis*.
- B: *A. segetum* GV grown in *A. segetum*.
- C: progeny virus from *N. pronuba* infected with *A. segetum* (E) NPV.
- D: *A. segetum* (E) NPV grown in *A. exclamationis*.
- E: *A. segetum* (E) NPV grown in *A. segetum*.
- F-J: *M. brassicae* NPV grown in *N. pronuba* (F), *P. gamma* (G), *A. exclamationis* (H), *A. segetum* (I), *M. brassicae* (J).
- K: λ Phage DNA EcoRI digest.

- There may, therefore, have been some A. segetum (E) NPV replication in these larvae. Neonate N. promuba larvae inoculated with A. segetum (E) NPV had died of infection by an NPV with I.B.'s morphologically similar to those of the inoculum and clearly distinct from CPV I.B.'s in appearance and cellular location (Figure 5.3). It was concluded that N. promuba was possibly susceptible to A. segetum (E) NPV but this was not confirmed by identification of this virus in infection progeny.

The possible origin of the N. promuba CPV infection of the older larvae is discussed in Section 2(b). This virus isolate had a ds RNA profile identical with that of CPV's produced in N. promuba and M. brassicae larvae inoculated with P. gamma CPV. The mobilities of the genome segments from this N. promuba CPV were compared with those of other CPV types on three electrophoretic gel systems (Appendix 1).

When A. segetum GV was fed to neonate N. promuba larvae, death often occurred, the corpses containing low to moderate numbers of GV I.B.'s, often mixed with bacteria. Typical symptoms of virus infection, such as liquefied corpses, were not found. When older larvae were inoculated with 10^8 - 10^9 I.B.'s, they showed no overt signs of infection, so it was not possible to identify the progeny virus in this case. It was concluded that, although low levels of GV replication may occur, N. promuba is not very susceptible to A. segetum GV infection.

On one occasion when P. rapae neonates were inoculated with A. segetum GV, considerable GV mortality occurred. However, the control larvae were also infected suggesting that contamination with P. brassicae GV may have been the cause of infection. P. rapae neonates are very susceptible to infection by this virus (Chapter 6), which was being regularly handled in the laboratory at the time of this assay. When the assay was repeated

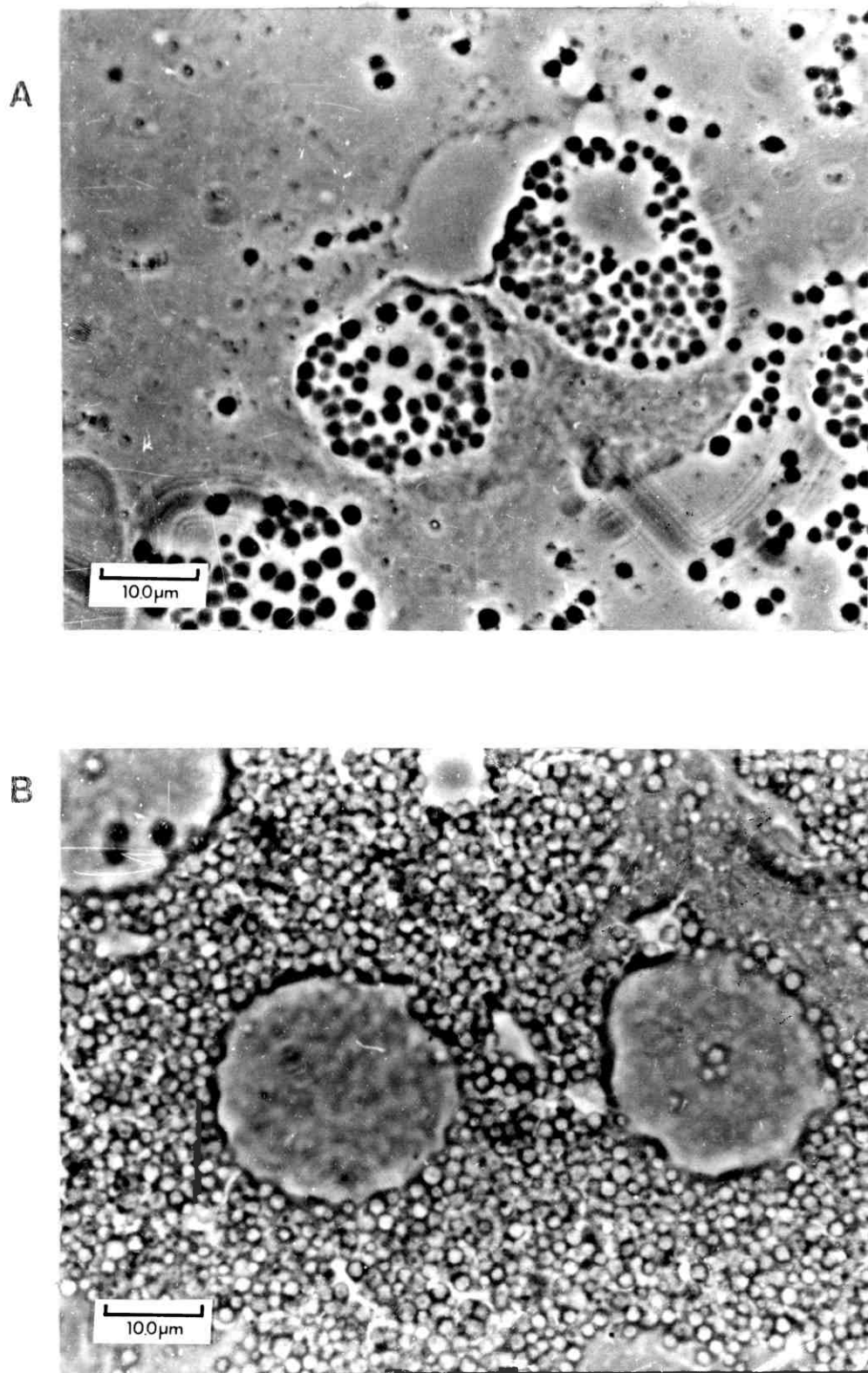


Figure 5.3. Phase contrast microscopy of smeared neonate larvae.
 A: *N. pronuba* inoculated with *A. segetum* (E) NPV, showing NPV IB's in nuclei,
 B: *P. gamma* inoculated with *P. gamma* CPV, showing CPV IB's in cytoplasm.

using sterile rather than washed plastic-ware, there were no virus deaths. These observations are interesting because similar events may explain the apparent transmission of an unknown cutworm GV to P. rapae described by Smith et al. (1964) (see Chapter 1, Section 5).

b) Cytoplasmic polyhedrosis viruses

Progeny viruses, from experiments where larvae had been inoculated with P. gamma CPV, were identified by electrophoresis of the genome segments on 3% acrylamide gels (Chapter 2). All five non-homologous species inoculated with P. gamma CPV as neonates had shown CPV infections (Table 6). In three of these cases the infection was repeated in older larvae and the progeny virus identified. The progeny CPV from inoculation of P. rapae larvae with P. gamma CPV, had the same genome segment profile as the inoculum, (Figure 5.4). Both M. brassicae and N. pronuba larvae, however, were infected with a CPV identified as N. pronuba CPV. This virus had the same segment profile as the CPV produced in N. pronuba following inoculation with A. segetum (E) NPV (Section 2(a)). When the genome segments of the preparation produced in M. brassicae were separated on a 10% polyacrylamide gel, faint bands were seen which corresponded to those of the P. gamma CPV profile (Figure 5.5). In this case it appeared that some P. gamma CPV replication had occurred, as the original inoculum would not have been present in sufficient quantity to be detected.

It was considered unlikely that the N. pronuba CPV was a contaminant of both P. gamma CPV and A. segetum (E) NPV inocula. When a large quantity of P. gamma CPV ds RNA was separated by electrophoresis on a 10% acrylamide gel two very faint contaminant bands appeared, but these did not correspond in mobility to any N. pronuba CPV genome segments

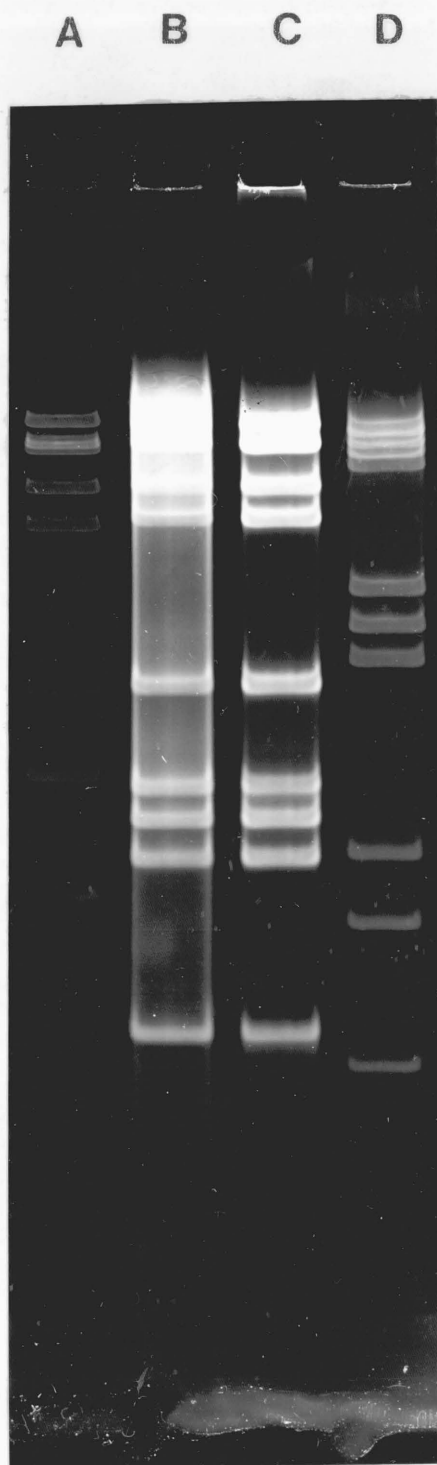


Figure 5.4. Electrophoresis of CPV inocula and progeny RNA segments on 3% acrylamide gel.

Key:

- A: *B. mori* CPV.
- B: *P. gamma* CPV grown in *P. gamma*.
- C: *P. gamma* CPV grown in *P. rapae*.
- D: Progeny virus from *N. pronuba* infected with *A. segetum*
- (E) NPV.

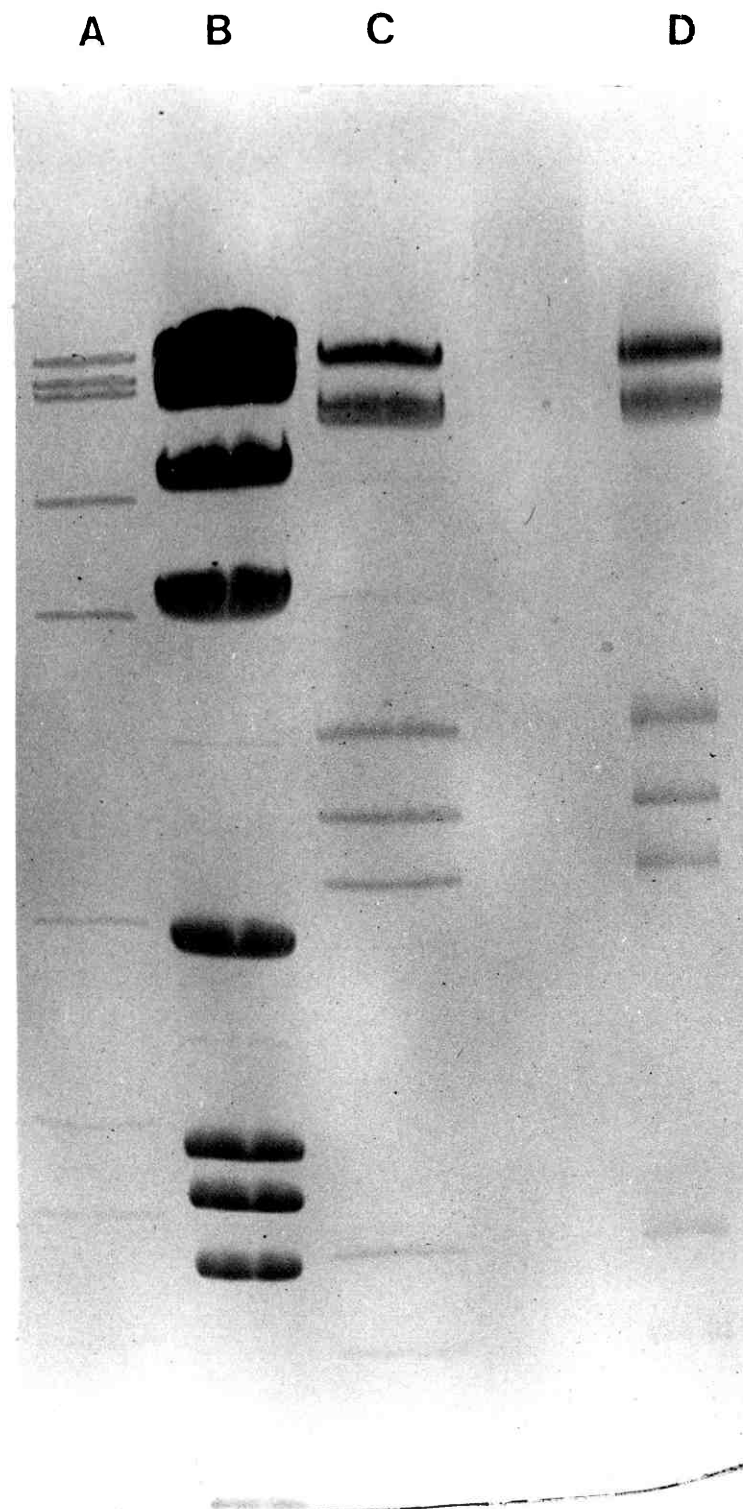


Figure 5.5. Electrophoresis of CPV inocula and progeny RNA segments on 10% acrylamide gel.

Key: A: *B. mori* CPV.
 B: *P. gamma* CPV.
 C: *M. brassicae* inoculated with *P. gamma* CPV.
 D: *N. pronuba* inoculated with *P. gamma* CPV.
N.B. gel stained in 0.005% toluidine blue in H₂O.

(Figure 5.5). Similarly, no contaminant bands were found in the A. segetum (E) NPV restriction endonuclease fragment profile (Chapter 3; Figure 5.2), and no CPV I.B.'s were detected in this inoculum by phase contrast microscopy. It would be difficult, in any case, to explain how both inocula became contaminated when they were prepared at different times and in different species. It seemed probable that the N. promuba CPV originated from one batch of field collected N. promuba adults. M. brassicae larvae presumably were contaminated with this CPV at the time of the progeny test inoculation, as there were no signs of CPV infection either in the main M. brassicae stocks or in M. brassicae larvae used in other virus progeny tests.

Infectivity tests in which N. promuba and M. brassicae neonates were dosed with P. gamma CPV gave dose-related mortality with LD50's of less than 50 I.B.'s in both cases, and no infections in the control insects (Chapter 6). These results are not consistent with mortality due to a virus contaminating the insects, suggesting that the neonates died of infection produced by the inoculum virus, P. gamma CPV.

(3) Susceptibility of M. brassicae to three European isolates of A. segetum NPV

M. brassicae neonates were not susceptible to infection by oral inoculation with A. segetum (E) NPV (Table 6). In contrast Burgerjon et al. (1975) and Lipa et al. (1972), had previously reported that M. brassicae larvae were susceptible to infection by A. segetum (F) NPV and A. segetum (P) NPV respectively, although in neither case was the progeny virus identified. Samples of these virus inocula were obtained (Chapter 2) and, after passaging at the GCRI in A. segetum, were tested for infectivity to M. brassicae neonates. A. segetum (F) NPV and

A. segetum (E) NPV which were biochemically indistinguishable (Chapter 3), were each tested twice in M. brassicae larvae but on no occasion was there death by virus infection, or I.B. production in larval tissue.

A. segetum (P) NPV showed limited production of I.B.'s in a few M. brassicae larvae, but no 'typical' virus deaths. It was not possible in this case to produce sufficient progeny virus for identification.

(4) Discussion

M. brassicae NPV had the widest host range of the baculoviruses tested, infecting all six of the noctuid species in three subfamilies. In all examples the progeny virus was identified as M. brassicae NPV by restriction endonuclease analysis. Previous studies have also noted the wide host range of this virus (Groner, 1978; Vlak and Groner, 1980; Burgerjon et al., 1975). In the present study it did not infect P. rapae, and previous studies have suggested that its host range is limited to noctuids (Vlak and Groner, 1980). The susceptibility of L. oleracea to M. brassicae NPV has not been previously shown, and is of interest because both L. oleracea and M. brassicae are glasshouse pests. Control of the former species by a GV has been considered, but in that case the virus was not very infectious (Crook et al., 1982).

The host ranges of A. segetum (E) NPV and A. segetum GV were limited to the subfamily noctuinae of those tested (Table 6). A. segetum (E) NPV did not infect M. brassicae as had been suggested by Burgerjon et al. (1975). When Croizier (1978; Pers. comm.) repeated the infection of M. brassicae larvae with the A. segetum (F) NPV inoculum used by Burgerjon, he found that the progeny virus was M. brassicae NPV. A. segetum (P) NPV caused a limited infection in M. brassicae larvae,

however, as Lipa et al. (1972) had suggested. In neither case was there confirmation of true cross-transmission by progeny identification.

A. segetum GV may infect N. pronuba at high doses but more studies are needed to confirm this. It is not known if this virus isolate was the same as an A. segetum GV isolate from Poland, which did not infect N. pronuba (Lipa and Ziemnicka, 1972).

P. gamma sNPV infected only the homologous host while P. brassicae GV infected only P. rapae. In the latter case, however, no other species from the homologous family were tested. The results only partly confirm the idea that mNPV's have wider host ranges than sNPV's and GV's (Chapter 1, Section 5). Although M. brassicae NPV had the widest host range of the baculoviruses, A. segetum (E) NPV and A. segetum GV appeared to have the same host range. None of the baculoviruses infected species from the alternative lepidopteran family tested. P. gamma CFV had a wider host range by this criterion, as it infected P. rapae in addition to the homologous host. This phenomenon, of cross-infectivity to non-homologous families, has been reported more often for CFV's than for baculoviruses (Chapter 1, Section 5).

P. gamma CFV appeared to infect the non-plusiine noctuids which were tested, but the progeny viruses from these infections were N. pronuba CFV at least in some cases. This example emphasised the importance of progeny virus identification in cross-transmission experiments.

The significance of virus host range for natural virus ecology and microbial control of insect pests, is dependent on their absolute infectivity in the different hosts. This is examined in the next Chapter.

CHAPTER 6 STUDIES OF VIRUS INFECTIVITY

<u>CONTENTS</u>	<u>PAGE</u>
(1) LD50 ASSAYS	180
(2) LT50 CALCULATIONS	186
(3) VIRUS PRODUCTION ASSAY	188
(4) DISCUSSION	189

CHAPTER 6

STUDIES OF VIRUS INFECTIVITY

Survival of an insect virus in the environment depends on the relationship between the rates of virus production and degradation. Properties of the virus which influence virus production include infectivity, the period between infection and virus production, and the quantity of infective virus produced in each host. In this chapter the first two of these properties were examined in comparison with the host ranges and I.B. structures of the different viruses. The production of virus in individual larvae was examined in one case.

Neonate larvae were used in tests of the median lethal doses (LD50's) and median lethal times (LT50's) of the viruses. Direct comparisons between species were possible using this standard larval stage. Neonate larvae are usually the most susceptible to infection, larvae becoming less susceptible with age at rates which vary in different species (Chapter 1, Section 6(a)). In terms of both virus ecology and insect control, infection of this stage is of prime importance, as discussed in Chapter 1, Section 7(b).

(1) LD50 Assays

The method of LD50 assay is described in Chapter 2. With baculovirus infections, daily mortality assessment continued until five days had passed with no further mortalities. However, in CFV infections small numbers of infected larvae remained alive after five to six weeks. These had not developed beyond first or second instar and could be clearly distinguished from uninfected larvae both in appearance and by

examination of dried larval smears. These larvae were considered moribund and were added to the number which died for the purpose of calculating percentage mortality.

The dosage-mortality responses were analysed using a maximum likelihood procedure (MLP program: G. J. S. Ross, Rothamsted Experimental Station, Hertfordshire, U.K.), to obtain LD50's and other parameters of the responses. The LD50's, fiducial limits and slopes are shown in Table 7. In all cases the computed dosage-mortality line was a good fit of the data (heterogeneity χ^2 not significant, $p > 0.05$). Preliminary assays, which were done to estimate the order of magnitude of the LD50's were in reasonable agreement with the final assay results given in Table 7.

The results of LD50 assays can vary several fold even when using a standard virus preparation and the same insect stock (Dulmage, 1973). Small differences in LD50's will not, therefore, be meaningful. Although not strictly correct, a useful approximation is to consider LD50's significantly different if their fiducial limits do not overlap. This approach was also used by Payne *et al.* (1981). For comparison the LD50 values are given to two significant figures in Table 8.

The slopes of the dosage-mortality response can also show considerable variation under standard conditions (Dulmage, 1973). The values given in Table 7 for the slopes of baculovirus dose-mortality assays, span the range usually found when the larval population is not artificially selected for homogeneity (Chapter 1, Section 6(a)). CPV infections exhibited a similar range of slopes. The 95% confidence limits of the slopes ($\pm 2 \times$ standard error) overlapped considerably between tests. The lack of observable pattern with respect to the

Table 7 Complete Results of LD50 Assays

<u>Virus</u>	<u>Host</u>	<u>LD50*</u>	<u>95% Fiducial Limits*</u>		<u>Slope</u>	<u>Standard Error of Slope</u>
<u>M. brassicae</u> NPV	<u>A. segetum</u>	2,100	1,240	5,350	0.95	0.20
	<u>A. exclamationis</u>	10,700	1,510	$10^{8.83}$	0.70	0.25
	<u>N. promba</u>	10.2	5.34	18.5	1.69	0.36
	<u>M. brassicae</u>	21.3	12.7	36.9	1.41	0.22
	<u>L. oleracea</u>	203	134	391	1.39	0.25
	<u>P. gamma</u>	3.49	0.924	6.58	0.90	0.20
<u>A. segetum</u> (E) NPV	<u>A. segetum</u>	10.5	6.86	14.9	1.29	0.15
	<u>A. exclamationis</u>	423	234	958	0.97	0.17
	<u>N. promba</u>	303	137	675	1.28	0.42
<u>A. segetum</u> (F) NPV	<u>A. segetum</u>	23.3	14.6	37.2	1.34	0.25
<u>A. segetum</u> (P) NPV	<u>A. segetum</u>	354	198	930	0.91	0.14
<u>P. gamma</u> sNPV	<u>P. gamma</u>	0.966	0.100	2.14	1.27	0.34

continued ...

Table 7 continued

<u>Virus</u>	<u>Host</u>	<u>LD50*</u>	<u>95% Fiducial Limits*</u>		<u>Slope</u>	<u>Standard Error of Slope</u>
<u>A. segetum</u> GV	<u>A. segetum</u>	10,600	5,960	24,700	1.10	0.21
	<u>A. exclamationis</u>	1,170,000	874,000	1,550,000	2.10	0.27
<u>P. brassicae</u> GV	<u>P. rapae</u>	12.0	7.22	18.5	1.86	0.24
<u>P. gamma</u> CPV	<u>N. pronuba</u>	14.2	8.41	22.0	1.83	0.38
	<u>M. brassicae</u>	47.6	21.7	280	0.95	0.29
	<u>P. gamma</u>	3.56	2.02	5.63	1.60	0.23

* expressed as number of I.B.'s, corrected to three significant figures.

Table 8 Comparative LD50's*

<u>Virus</u>	<u>Host Species</u>						
	<u>A. segetum</u>	<u>A. exclamationis</u>	<u>N. promuba</u>	<u>M. brassicae</u>	<u>L. oleracea</u>	<u>P. gamma</u>	<u>P. rapae</u>
<u>M. brassicae</u> NPV	2,100	11,000 ⁺	10	21	200	3.5	—
<u>A. segetum</u> (E) NPV	10	420	(300)	—	—	—	—
<u>A. segetum</u> (F) NPV	23						
<u>A. segetum</u> (P) NPV	350						
<u>P. gamma</u> sNPV	—	—	—	—	—	1.0	—
<u>A. segetum</u> GV	11,000	1,200,000	—	—	—	—	—
<u>P. brassicae</u> GV	—	—	—	—	—	—	12
<u>P. gamma</u> CPV	n.t.	n.t.	(14)	(48)	n.t.	3.6	n.t.

Key: * no. I.B.'s to 2 significant figures; ⁺ wide fiducial limits (Table 7)

() progeny virus not confirmed (Chapter 5); — not susceptible; n.t. dose-mortality not tested.

different species suggested that the variation in slopes was due to variation in test conditions and had no biological significance.

When the LD50 values were compared (Table 8), it was apparent that there were considerable variations in the LD50's of each virus for its homologous and heterologous hosts, and also between different viruses in each host. P. gamma was very susceptible to the three viruses tested in this species, which had LD50's of < 5 I.B.'s. Conversely, the three viruses assayed in A. exclamationis were each less infective in this species than in the other species tested. M. brassicae NPV showed the largest variation in infectivity, the LD50 in A. exclamationis being 3,000 fold greater than that in P. gamma.

In six of the eight viruses tested, the LD50 for the homologous host was less than 25 I.B.'s. The exceptions were A. segetum (P) NPV and A. segetum GV. There is evidence that homologous GV infections of other noctuids have high LD50's, while the homologous NPV infections of those species have relatively low LD50's (Chapter 1, Section 6(a)). A preliminary study of the infectivity to L. oleracea neonates of the homologous GV infection, suggested that noctuid GV infections may have high LD50's where no homologous NPV has been reported. The virus inoculum for this test was obtained from N. E. Crook (G.C.R.I.). Mortalities of 17% and 57% were found at doses of 10^3 and 10^4 I.B.'s per neonate respectively. The LD50 appeared to be about 10^4 I.B.'s which would be in agreement with extrapolation of the data for second, third, fourth and fifth instars (Crook et al., 1982) to first instars.

(2) LT50 Calculations

LT50 values were calculated from the daily dose-mortality figures, summed over all doses. This is acceptable because LT50 does not appear to depend on dose (Chapter 1, Section 6(b)) except at extreme doses. The hypothesis of constant LT50 with dose variation was further tested by analysis of LT50 at high and low doses of the dose-mortality assay, in three assays. In these examples, one of which was a CPV infection, the LT50 at the high and low doses varied by less than 5% despite dose variation of up to 100 fold.

The LT50's were calculated by the simple graphical method described by Litchfield (1949). In the case of baculoviruses, the maximum mortality in the dose-mortality assay was considered to cover the complete time-mortality response (Chapter 1, Section 6(b)). In the case of CPV infections, however, the assay was stopped before mortality was complete (Section 1). Therefore, the data filled a truncated time-mortality response with the observed mortality taken as a percentage of the expected maximum mortality.

The LT50 results are given in Table 9. The fiducial limits were calculated directly from the slope of the probit/log-time relationship (Litchfield, 1949) and did not take into account the heterogeneity of data points around this straight line. When replicated assays were compared, larger differences in LT50 were found than would be suggested from the fiducial limits (Table 9). This may also be the result of systematic differences between assays, such as the mean age of test larvae or the exact time of daily sampling. Variable virus acquisition times (Chapter 1, Section 6(b)), were probably not important in this respect, as in most cases feeding was complete in less than half the 24 hour period allowed (Chapter 2).

Table 9 LT50 Results

<u>Virus</u>	<u>Host</u>	<u>LT50</u> <u>days</u>	<u>95% Fiducial Limits</u>		<u>Slope</u>
<u>M. brassicae</u> NPV	<u>A. segetum</u>	12.2	11.9	12.5	24.7
	<u>A. exclamationis</u>	16.6	15.4	17.9	18.5
	<u>N. pronuba</u>	8.7	8.4	9.0	18.5
	<u>M. brassicae</u>	9.8	9.4	10.2	14.7
	<u>L. oleracea</u>	10.4	9.9	11.0	13.5
	<u>P. gamma</u>	8.7	8.5	8.9	18.5
<u>A. segetum</u> (E) NPV	<u>A. segetum</u> (1)	9.8	9.5	10.1	17.5
	<u>A. segetum</u> (2)	9.0	8.8	9.2	18.0
	<u>A. exclamationis</u>	10.4	9.8	11.0	12.0
	<u>N. pronuba</u>	8.6	8.1	9.1	14.1
<u>A. segetum</u> (F) NPV	<u>A. segetum</u>	8.1	7.8	8.5	14.6
<u>A. segetum</u> (P) NPV	<u>A. segetum</u>	11.2	10.5	11.9	13.5
<u>P. gamma</u> sNPV	<u>P. gamma</u>	6.0	5.9	6.1	23.5
<u>A. segetum</u> GV	<u>A. segetum</u> (1)	10.0	9.5	10.5	11.2
	<u>A. segetum</u> (2)	8.4	7.7	9.1	8.1
	<u>A. exclamationis</u>	8.1	7.8	8.4	12.1
<u>P. brassicae</u> GV	<u>P. rapae</u>	5.5	5.4	5.6	23.0
<u>P. gamma</u> CPV	<u>N. pronuba</u>	14.1	13.4	14.9	9.1
	<u>M. brassicae</u>	20.1	17.2	23.6	3.8
	<u>P. gamma</u>	15.5	13.9	17.3	4.1

Key: (1), (2) - replicated assays

The LT50's for baculovirus infections varied from 8.1 to 12.2 days with three exceptions. A. exclamationis infected with M. brassicae NPV had a considerably greater LT50 of 16.6 days, while the infections of P. rapae with P. brassicae GV and P. gamma with P. gamma sNPV had shorter LT50's of 5.5 and 6.0 days respectively. Amongst the 'main group' of baculovirus LT50's there was no observable correlation of LT50 with virus types or host species. However, there was a positive relationship between LT50 and LD50 in several cases, notably among infections by M. brassicae NPV. The LT50's of CPV infections were markedly greater than all but one of the baculovirus LT50's. There was considerable variation in the slope of the time-mortality response, with the CPV infections having the most shallow slopes.

(3) Virus Production Assay

In order to count the numbers of viral I.B.'s produced in neonate larvae, it was necessary to macerate the larvae, individually, in a ground-glass tissue grinder. By counting samples of the macerate the total number of I.B.'s in each larva could be calculated. This method is suitable for baculovirus infections where it is assumed that no I.B.'s are lost before larval death (Payne, 1982). It would not be suitable for CPV infections where I.B.'s are excreted continuously throughout the latter stages of infection (Payne, 1981(b)).

Methods for counting impure I.B. preparations were compared. Haemocytometer counting was unsuitable, as larval debris could not be distinguished from I.B.'s. NPV I.B.'s could be counted in stained dry smears (Wigley, 1980), but this was not suitable for GV I.B.'s (Chapter 2). An alternative method for counting GV I.B.'s was tried, using

droplets of virus suspension mixed with latex spheres which had been sprayed onto an electron microscope grid. However, although purified GV suspensions could be counted by this method, it was not suitable for counting GV I.B.'s in larval homogenates. I.B.'s were not clearly distinguishable, due to clumping and the presence of larval debris. GV I.B. production in neonates was not assayed due to these technical problems.

The production of A. segetum (E) NPV I.B.'s was examined in the homologous host using stained, dried smears. Corpses of eight neonates were collected from an LD50 assay and the NPV I.B.'s counted as described by Wigley (1980).

Three purified NPV I.B. preparations were also counted by this method and the results were 58%, 56% and 71% of haemocytometer estimates of the same preparations. The difference might have been due to I.B. losses during staining. To compensate for this effect, the results of I.B. counts in A. segetum neonate homogenates were multiplied by 1.62 (the reciprocal of the mean fraction of I.B.'s lost).

The mean ($\pm$ 95% confidence limits) of the number of A. segetum (E) NPV I.B.'s produced in A. segetum neonate corpses was $1.36 (\pm 0.45) \times 10^6$.

(4) Discussion

The low LD50's, expressed as numbers of I.B.'s, found in most of the viral infections of homologous neonates (Table 8), confirmed the pattern of reports in the literature (Chapter 1, Section 6(a)). Where LD50's had been estimated previously by other workers, there was reasonable agreement. Payne et al. (1981) assayed P. brassicae GV in P. rapae

neonates and reported an LD50 of 5 I.B.'s compared with 12 in the present study. Evans (1981a) found an LD50 of 7 I.B.'s for M. brassicae NPV in the homologous host, compared with 21 I.B.'s here.

Heterologous infections sometimes had higher LD50's than the equivalent homologous infection, but this was not always the case. M. brassicae NPV was highly infectious for N. pronuba and F. gamma neonates in addition to M. brassicae neonates. It was less infectious for A. segetum and A. exclamationis larvae. Similar comparative infectivity results were found by Groner (1978) and Vlak and Groner (1980) who examined the LC50's of M. brassicae NPV in second instars of several noctuid species. However, Groner (1978) found that A. exclamationis larvae were more susceptible than A. segetum larvae to M. brassicae NPV, in contrast with the results observed in the present study. The value of a virus for insect control will depend on its quantitative infectivity to alternative hosts, in addition to the host range. M. brassicae NPV would, on this basis, be suitable for use against neonate N. pronuba, F. gamma and possibly L. oleracea, in addition to the homologous host. However, it would not be as suitable as A. segetum (E) NPV for use against the two Agrotis spp.

F. gamma CPV had LD50's of less than 50 I.B.'s in all three species tested (although note the comments on progeny identification in Chapter 5). With this virus the LD50 was equal to the median infective dose (ID50), as there were no signs of infection in larvae which were not moribund or dead. This is not always the case with CPV infections. Bell and Kanavel (1976), for example, estimated the median infective concentration (IC50) of Pectinophora gossypiella CPV in neonates of the homologous host as 1000 fold less than the LC50.

Two exceptions to the pattern of low LD50's in homologous neonate infections were the noctuid GV's A. segetum GV and L. oleracea GV. There is no evidence that these viruses have alternative, more susceptible, host species. The limited number of studies on noctuid GV infectivity in the literature, supports the view that they are less infective to their homologous hosts, in terms of numbers of I.B.'s, than noctuid NPV's or many non-noctuid GV's (Chapter 1, Section 6(a)). It may be more meaningful, however, to convert these LD50's into equivalent numbers of potentially infective units, for example virus particles or nucleocapsids. This is considered in Chapter 8.

The other virus with a high homologous LD50 was A. segetum (P) NPV. Lipa et al. (1971) considered that this virus was the same as an NPV isolated from A. exclamationis larvae. There was evidence that A. exclamationis was more susceptible to this virus (Lipa et al., 1972) which may indicate that A. exclamationis is the natural host.

The LT50 estimates (Table 9) were similar in range to values given in the literature (Chapter 1, Section 6(b)). Payne et al. (1981) estimated the LT50 for the P. brassicae GV infection of P. rapae neonates as 5.7 days which is close to the value of 5.5 days in the present study. The LT50 for M. brassicae infected with M. brassicae NPV was higher than the value of 6.7 days given by Evans (1981(a)), which may be due to the use of different assay temperatures (20°C in the present study compared with 22°C). Similarly the LT50 for A. segetum GV in the homologous host was intermediate between values recalculated from Charpentier (1980) at 17°C and 22°C (Chapter 1, Section 6(b)). However, the short LT50 for P. gamma sNPV in P. gamma larvae contrasted with Harper's (1976) finding that plusiine sNPV's had longer LT50's than mNPV's. Also, the LT50 of

A. segetum GV in A. segetum was similar to that of A. segetum (E) NPV, rather than being longer as has been reported in other comparisons of GV's and NPV's from the same species (Chapter 1, Section 6(b)).

CPV infections had longer LT50's than most baculovirus infections, and also shallower probit mortality/log-time response slopes (Table 9). These differences may be related to the different infection pathways of these two virus types (Chapter 1, Section 4(e)). In CPV infections, only the midgut epithelium is involved, and death is caused by starvation as a result of reduced absorption. This, combined with mechanisms for partial recovery by gut cell regeneration (Payne, 1981(b)), results in a long and variable period before death. In contrast, baculovirus infections of Lepidoptera cause death by inactivation of a variety of larval tissues after the initial replication cycle. Death occurs, therefore, after a short, less variable, period. The LT50 has a different significance for progeny production in CPV and baculovirus infections. In the latter case the period before death is a 'latent' period, with no progeny virus release. CPV's however, release infective virus into the environment from an early stage of infection (Chapter 1, Section 4(e)).

The estimated production of A. segetum (E) NPV I.B.'s by infection of A. segetum neonates was, at 1.4×10^6 , lower than the figure of 2×10^7 estimated by Evans (1981(b)) for I.B. production by M. brassicae NPV in the homologous host. It is probable that the total number of viral I.B.'s produced in each larva, depends on the quantity of precursors required for each I.B. This quantity will be related to the mean I.B. volume for that virus. The four fold difference in mean volume between M. brassicae NPV I.B.s and A. segetum (E) NPV I.B.'s (Chapter 4), may, therefore, account for a proportion of the difference in virus production

estimates given above. When virus inocula were produced in older larva, rough calculation showed that 10^2 - 10^3 fold more I.B.'s per larva were produced in GV infections than in NFV infections, which confirms the relationship between I.B. size and virus production.

The relationships between the parameters analysed in this Chapter and the design of virus I.B.'s, are discussed in the context of virus ecology in Chapter 8.

CHAPTER 7 INCLUSION BODY DISSOLUTION

<u>CONTENTS</u>	<u>PAGE</u>
(1) <u>IN VITRO</u> DISSOLUTION	195
(2) DISSOLUTION OF RADIOLABELLED I.B.'S <u>IN VIVO</u>	196
(3) ELECTRON MICROSCOPIC EXAMINATION OF DISSOLUTION <u>IN VIVO</u>	202
(4) DISCUSSION	209

CHAPTER 7

INCLUSION BODY DISSOLUTION

Baculovirus I.B.'s dissolve in the gut of lepidopterous larvae, releasing virus particles which can attach to the midgut epithelium (Chapter 1, Section 4). In this Chapter, the extent and rate of dissolution of four baculoviruses were examined in vitro (Section 1) and in vivo (Section 2), to show whether differential release of virus particles influenced virulence or host specificity. A limited study of dissolution, by electron microscopy, was carried out to see if there were differences between two of the baculoviruses in the mode of dissolution, or the survival of virus particles in the gut lumen (Section 3).

(1) In Vitro Dissolution

I.B. dissolution was assayed spectrophotometrically in buffers at a range of pH values. Use of u.v. and visible light at different wavelengths was tried, and 650 nm wavelength light was found most suitable. At this wavelength, absorbance by alkaline dissolved I.B.'s was less than 5% of that by the equivalent number of intact I.B.'s. Both GV and NPV I.B.'s showed a linear relationship between I.B. concentration and absorbance at 650 nm, at the tested concentrations of up to 1 mg/ml. Therefore, the changing absorbance during I.B. dissolution could be related to the rate of dissolution. When absorbance was plotted against time, sigmoid curves were produced, similar in form to those described by Egawa and Summers (1972) (see Chapter 1, Section 4(a)). The exponential decay phase was used to calculate the dissolution half-life. This value varied when I.B.'s were dissolved in different buffer systems, the buffers' ionic strength

being particularly important. For example, P. brassicae GV dissolved rapidly in 0.1 M carbonate/bicarbonate buffer pH 9.7 (50% dissolution in less than 1 minute), but more slowly in the same buffer diluted to 0.05 M (50% dissolution in 7 minutes).

0.1 M sodium carbonate/bicarbonate was used as a constant ionic strength buffer for comparative dissolution studies. An equal volume of I.B.'s at a suitable concentration (about 1 mg/ml) was added to the buffer. Buffer pH did not alter as a result of this addition. The results of dissolution experiments using N. brassicae NPV, A. segetum (E) NPV, A. segetum GV and P. brassicae GV are shown in Figure 7.1. P. brassicae GV I.B.'s were susceptible to dissolution at lower pH than the other baculoviruses, all of which had similar responses, with A. segetum (E) NPV I.B.'s the most resistant.

To see if these differences were also found during in vivo dissolution, the two viruses showing the most extreme susceptibilities in vitro (A. segetum (E) NPV and P. brassicae GV) were compared in vivo.

(2) Dissolution of Radiolabelled I.B.'s In Vivo

The production, purification and counting of ^{32}P - and ^3H -labelled viruses were described in Chapter 2. Dissolution was assayed in third instar larvae of homologous and heterologous hosts, by macerating the larvae or larval gut contents, at intervals after feeding, and pelleting undissolved I.B.'s by low speed centrifugation.

Labelled virus was fed to larvae as a dried smear on cabbage leaf discs, prepared as described in Chapter 2 Section 9(a). In each case, sufficient virus was smeared on a 10 mm disc to give 200-1000 counts per

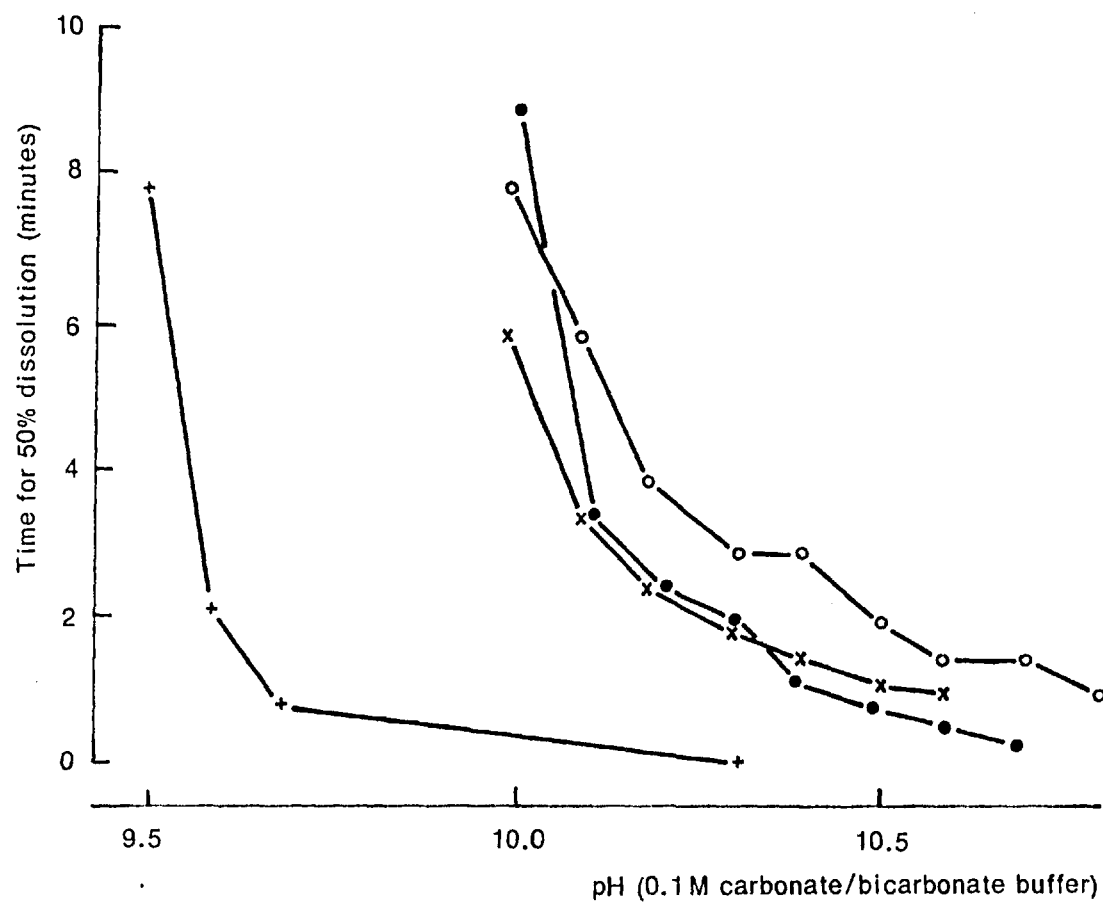


Figure 7.1. Alkaline dissolution of baculovirus IB's *in vitro*.

Key:-

- + *P. brassicae* GV
- x *M. brassicae* NPV
- *A. segetum* GV
- *A. segetum* (E) NPV

minute on each small feeding disc. Feeding took place in square compartmentalised dishes in which the leaf discs were placed on a 3% agar base. In all cases feeding was complete in less than five minutes after each larva started eating. Two to four larvae were sacrificed at each of the time intervals indicated in Figures 7.2 and 7.3. These were macerated whole (^{32}P -labelled virus), or the gut contents were removed and macerated (^3H -labelled virus), in 0.5 ml of 0.05 M Tris/Hcl pH 7.8 at 4°C. With both the GV and the NPV, undissolved I.B.'s were pelleted by centrifugation at 3,500 g for 20 minutes in 1 ml centrifuge tubes.

The counts in the supernatant and the pellet were used to calculate the fraction of I.B.'s which had dissolved.

When larval or gut content homogenates, which were free of labelled virus, were added to known quantities of labelled virus, quenching was found to be negligible. In the case of ^{32}P - and ^3H -labelled A. segetum (E) NPV, but not ^3H -labelled F. brassicae GV, the counts given by I.B. preparations after dissolution were approximately twice those before dissolution. This indicated that the I.B. protein matrix was having a quenching effect, and the counts given by pelleted NPV I.B.'s were corrected before estimating the fractional dissolution.

The results of dissolution experiments are given in Figures 7.2 and 7.3. Figure 7.2 shows the release of ^{32}P from A. segetum (E) NPV I.B.'s fed to A. segetum or M. brassicae. Complete dissolution appeared to take about three hours in both species, being quicker in M. brassicae.

When the location of the ^{32}P -labelled in A. segetum (E) NPV I.B.'s was examined by equilibrium sucrose gradient centrifugation of alkaline-dissolved I.B.'s, the label remained at the top of the gradient.

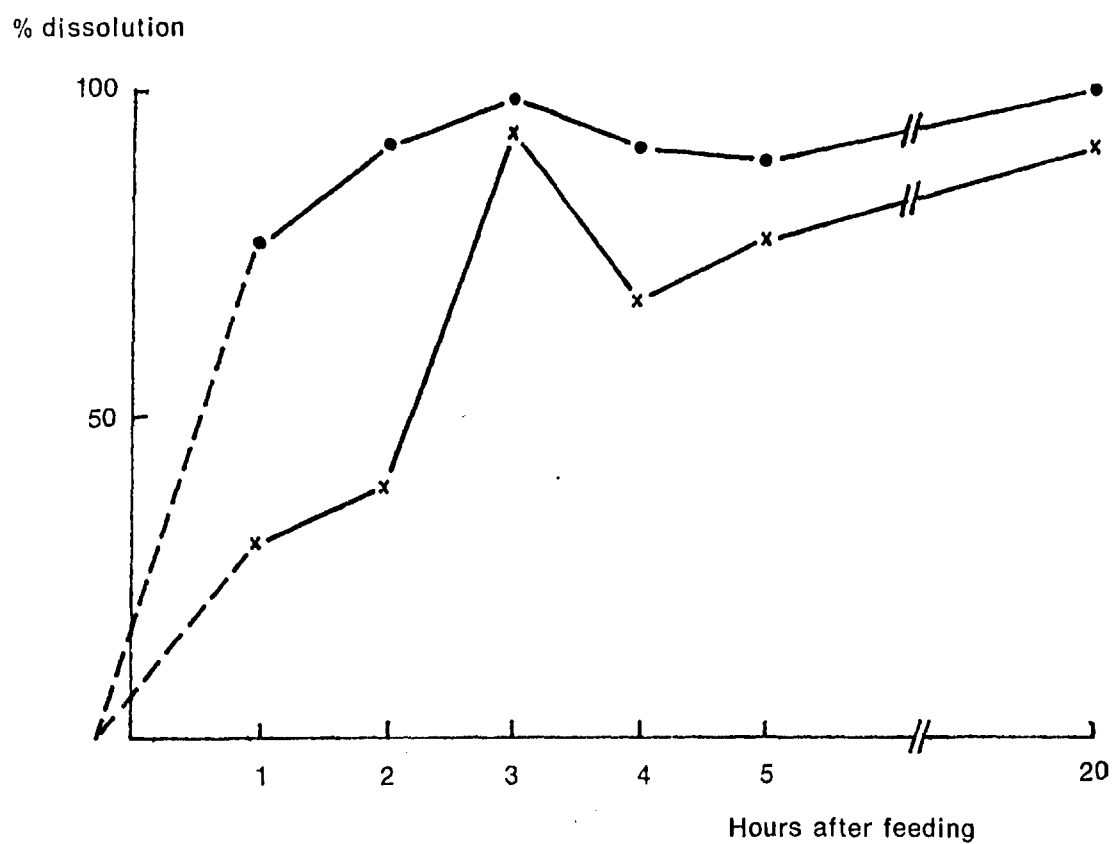


Figure 7.2. Dissolution of ^{32}P -labelled *A. segetum*(E) NPV IB's in vivo.

Key:-
 ● dissolution in *M. brassicae* larvae
 x dissolution in *A. segetum* larvae

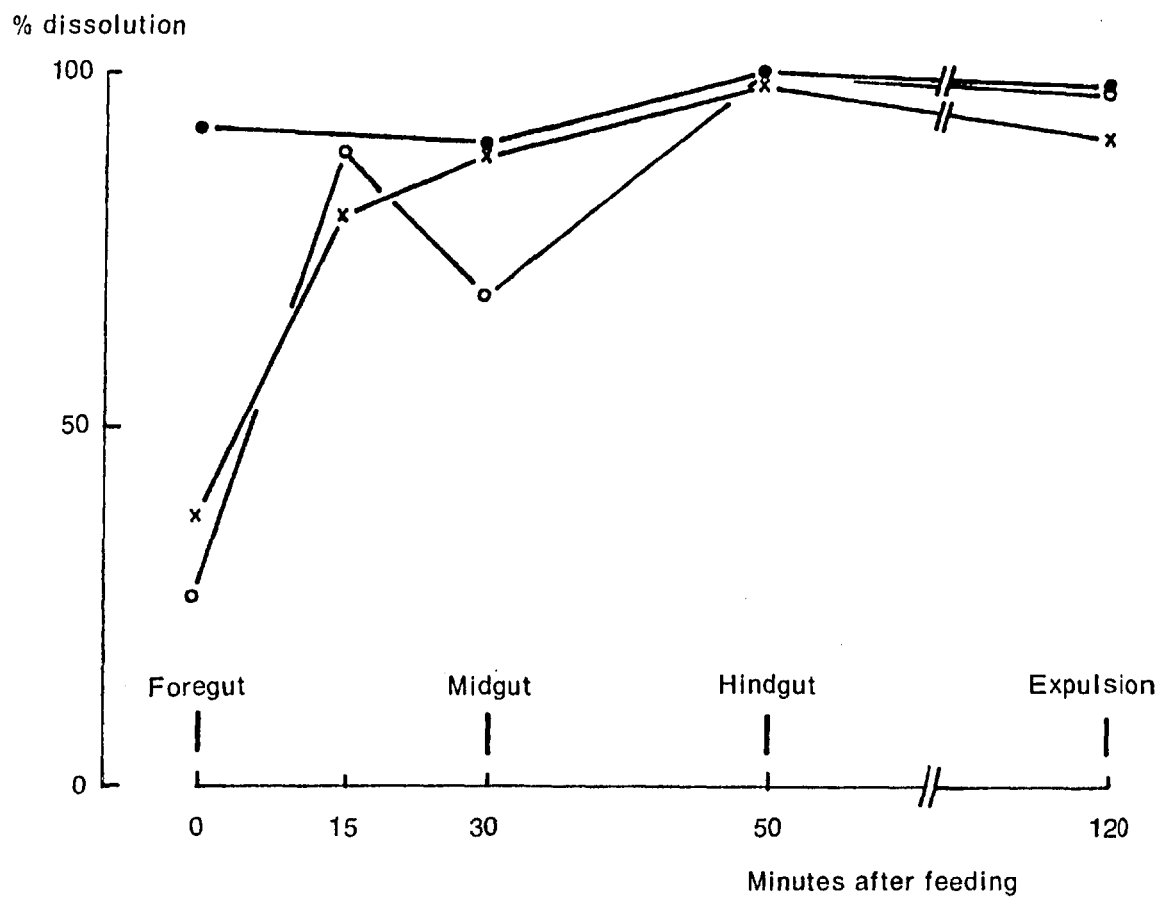


Figure 7.3. Dissolution of ^3H -labelled IB's *in vivo*.

Key:-
 ● *P. brassicae* GV in *P. rapae* larvae
 ○ *A. segetum* (E) NPV in *P. rapae* larvae
 ✱ *A. segetum* (E) NPV in *N. pronuba* larvae

It is known that I.B. matrix protein may be phosphorylated (Harrap and Payne, 1979). However, when the top fraction of the sucrose gradient was separated by SDS-discontinuous polyacrylamide gel electrophoresis, the label did not enter the 3% upper gel and was not associated with the band of I.B. matrix protein. Clearly the ^{32}P -label was not associated with the virus particles, but perhaps had been incorporated into a phospholipid component of the I.B.'s. Further experiments were done with ^3H -thymidine labelling of the virus preparations. In this case the label was associated with the virus particle band when the dissolved I.B. components were separated on isopycnic sucrose gradients.

Using ^3H -labelled virus preparations, dissolution was complete within 15 minutes in all cases (Figure 7.3). The positions of the leaf discs in the larval guts were noted before removal of the gut contents, and at this time they were usually between the foregut and midgut. The longer times noted for ^{32}P -labelled A. segetum (E) NPV may have been due to the different location of the radiolabel in this case. It is more likely that the results in Figure 7.3 show the true rate of virus particle release as the virus particles themselves were the site of labelling.

During the experiments with ^3H -labelled viruses, the gut epithelia and remainders of the larval bodies, were macerated and counted separately to see if uptake of the viruses could be detected. However, contamination by label from the gut contents, during the dissection process, prevented this. It was found that in all cases at least 50% of total counts were associated with the gut contents. This indicated that a large proportion of nucleocapsids remained in the larval gut and were not taken up by the gut epithelium.

(3) Electron Microscopic Examination of Dissolution In Vivo

Neonate M. brassicae were fed with M. brassicae NPV, and P. rapae neonates with P. brassicae GV or A. segetum GV. The viruses were either incorporated into diet or smeared on leaf discs. The preparation of these larvae for sectioning was described in Chapter 2. Sections were taken at 200 μ m intervals through the larvae, to examine I.B. dissolution in the different gut regions. These regions were defined by the morphology of the epithelial tissue, microvilli being present only on midgut epithelial cells.

Dissolution of I.B.'s began in the foregut and was nearly complete by the midgut which agrees with results in Section 2. Two extreme forms of dissolution were noted. In the first virus particles were released with little apparent breakdown of the I.B. matrix (Figure 7.4). In contrast, other I.B.'s lost most of the I.B. matrix, except for a surface layer, with little, or no, apparent virus particle release (Figure 7.5).

While most I.B.'s had dissolved before reaching the midgut, some M. brassicae NPV I.B.'s were found undissolved in the midgut. These were found between the peritrophic membrane and the microvilli (Figure 7.6). It was not clear how these I.B.'s broached the membrane, but having done so they seemed 'protected' from dissolution. In agreement with previous studies (Chapter 1, Section 4(c)), the peritrophic membrane appeared to be a barrier to virus particle movement in the case of M. brassicae NPV virus particles in M. brassicae larvae (Figures 7.6; 7.7). However some virus particles were seen in the vicinity of the microvilli (Figure 7.8).

Despite the large numbers of M. brassicae NPV virus particles seen in the lumen, no nucleocapsids were found within microvilli, suggesting

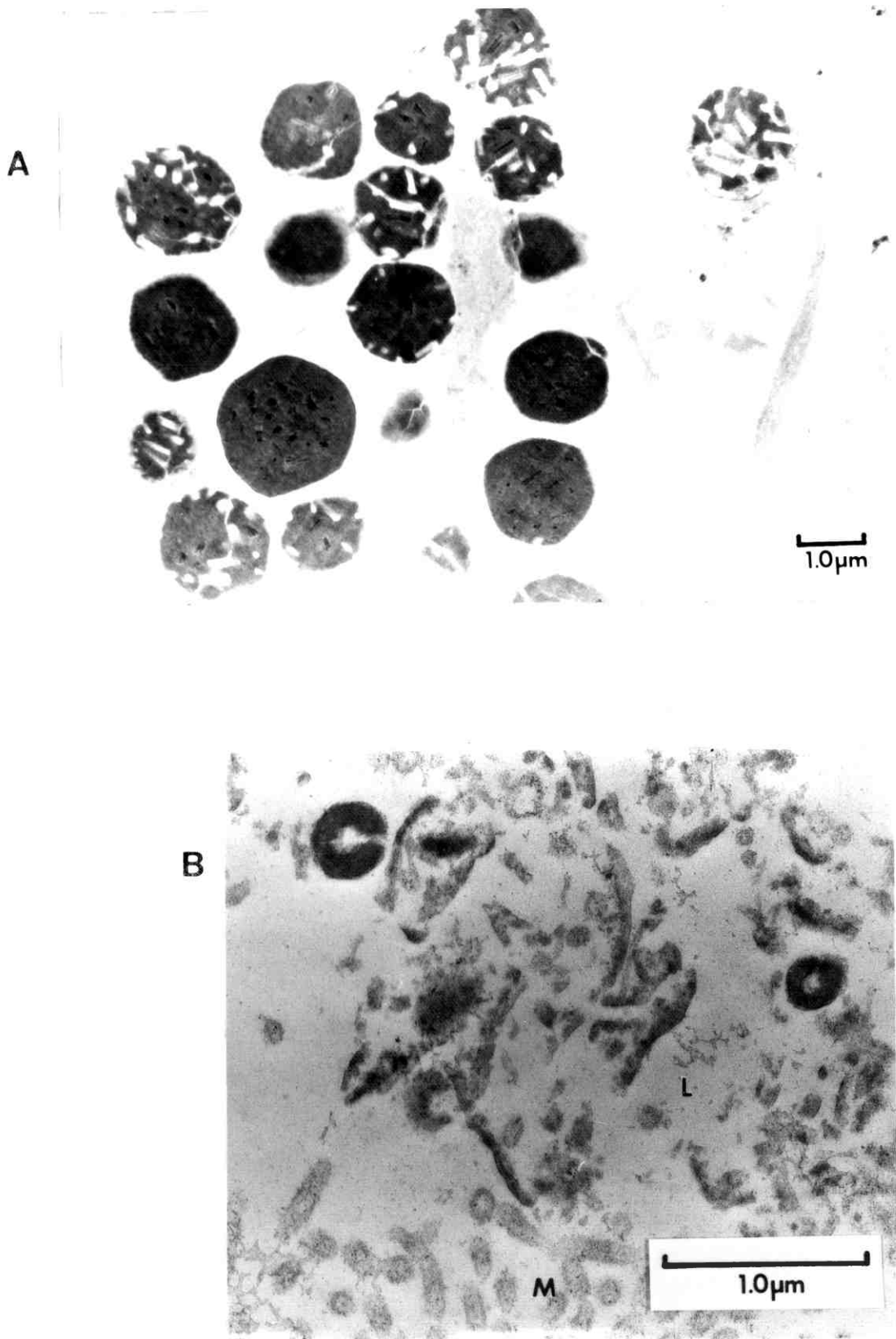


Figure 7.4. IB dissolution *in vivo*,

A: *M. brassicae* NPV in *M. brassicae* neonate foregut.

B: *A. segetum* GV in *P. rapae* neonate midgut.

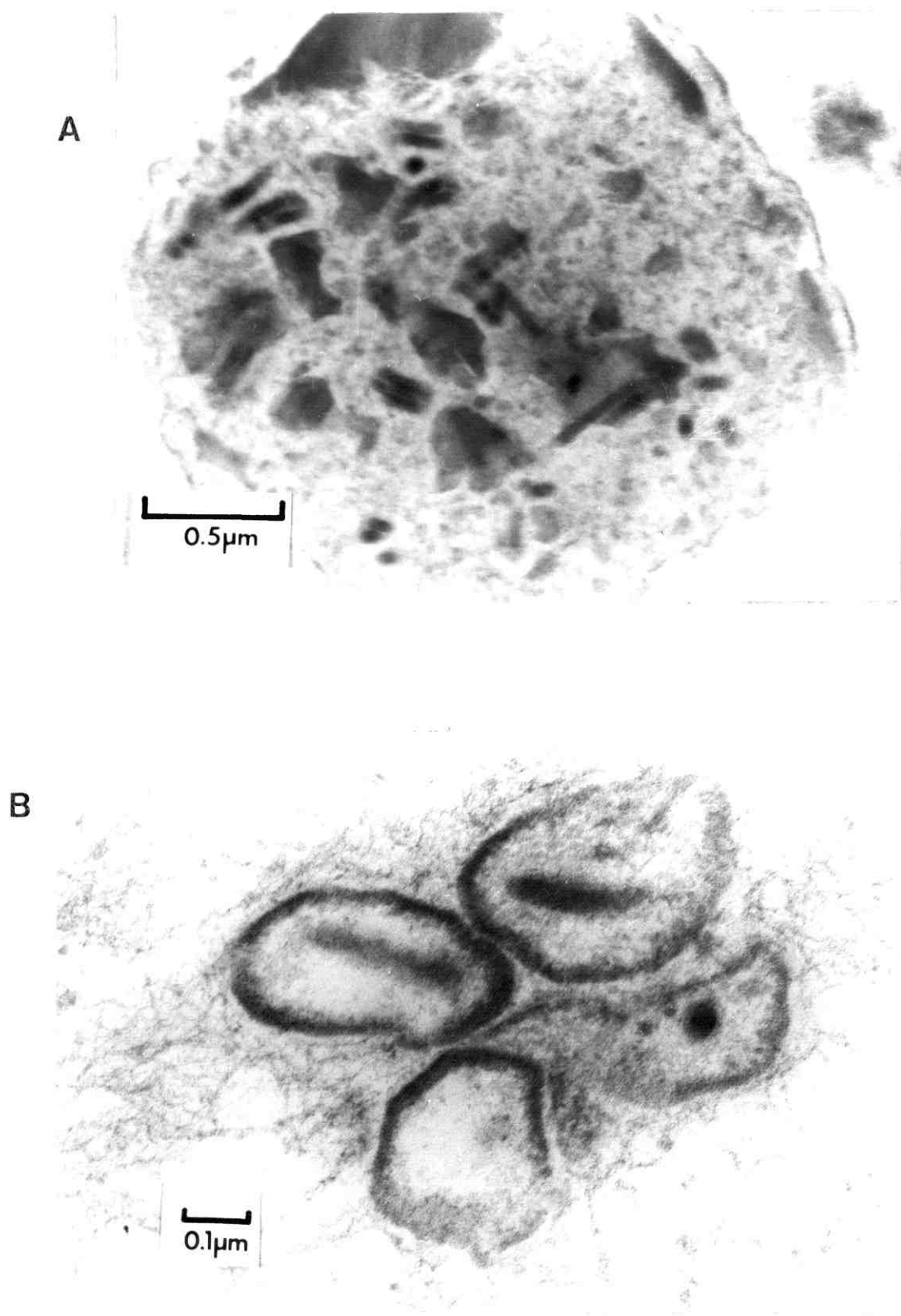


Figure 7.5. iB dissolution *in vivo*.

A: *M. brassicae* NPV in *M. brassicae* neonate midgut.

B: *P. brassicae* GV in *P. rapae* neonate midgut.

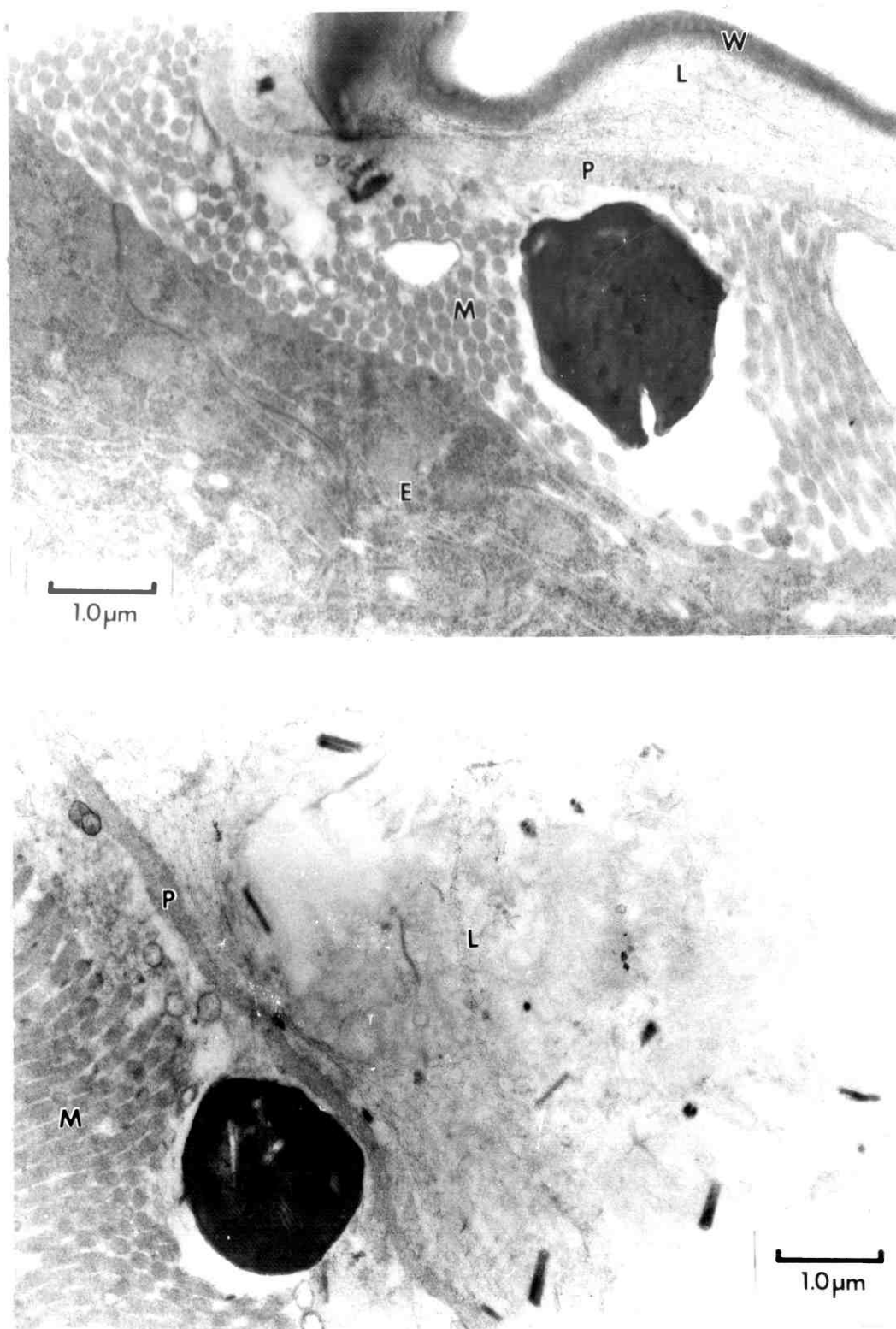


Figure 7.6. Midgut of *M. brassicae* neonates fed *M. brassicae* NPV, showing undissolved IB's.

Key: E: epithelial cells
M: microvilli
P: peritrophic membrane
L: lumen
W: plant cell wall

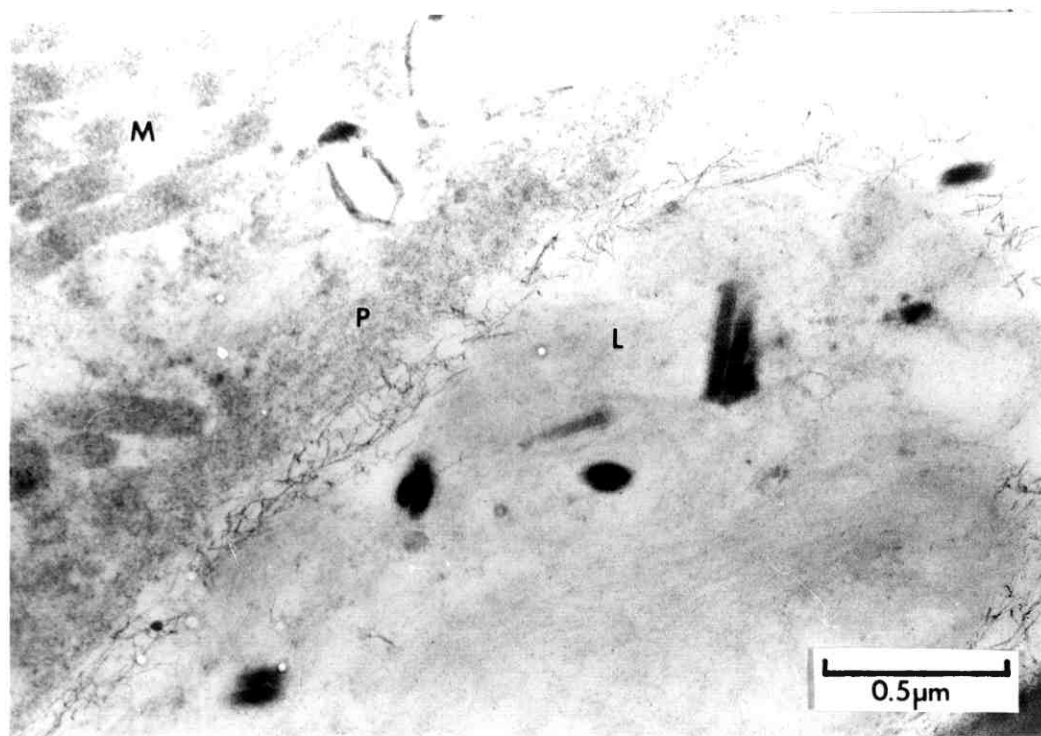
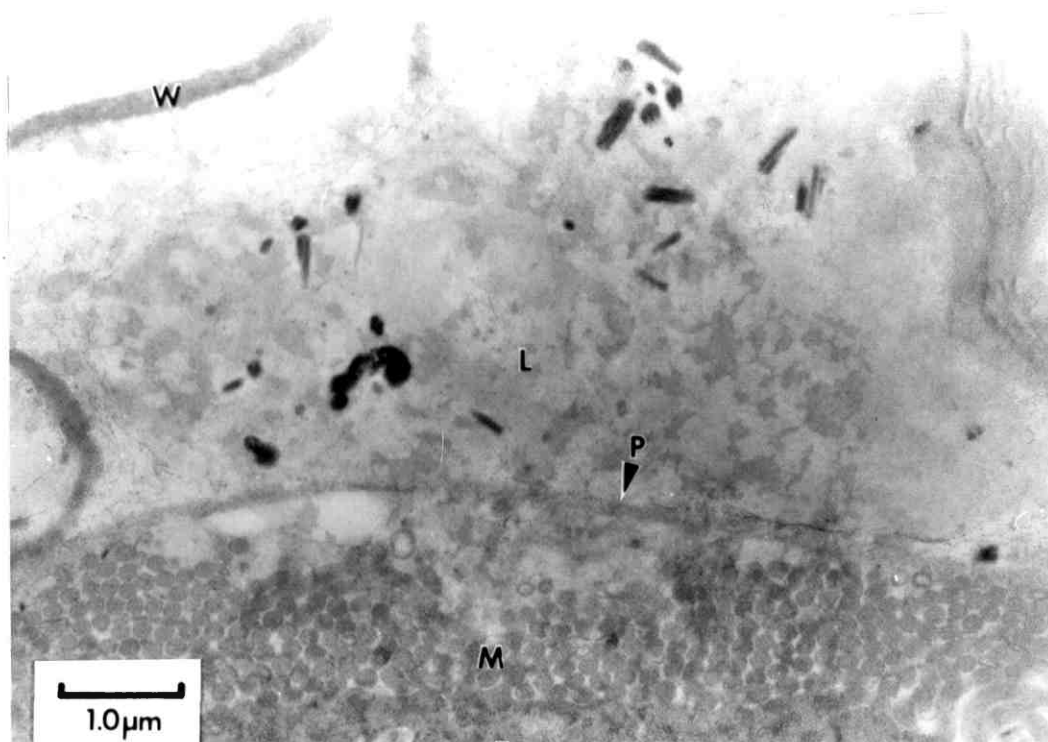


Figure 7.7. Midgut of *M. brassicae* neonates fed *M. brassicae* NPV; peritrophic membrane acting as a barrier to infection.

Key: as Figure 7.6.

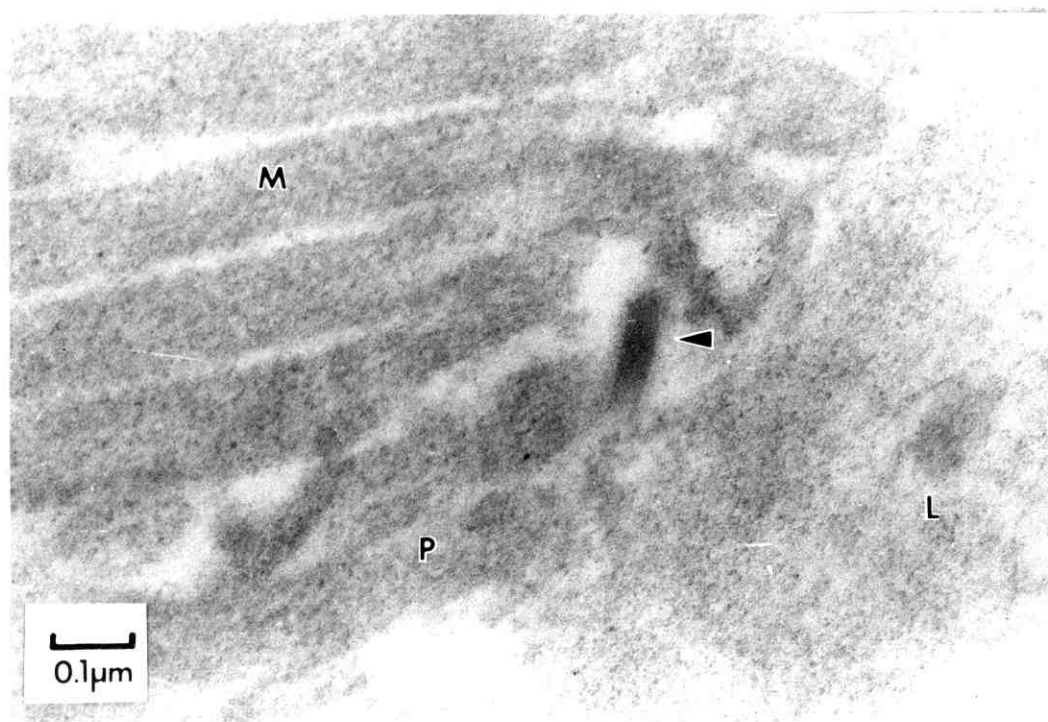
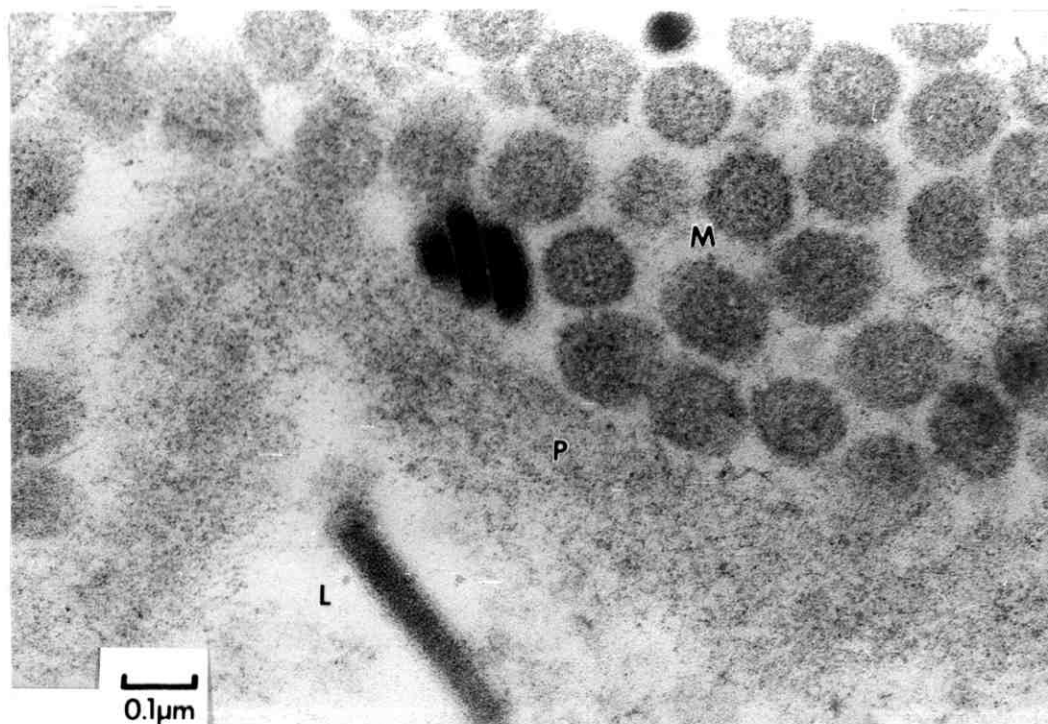


Figure 7.8. Midgut of *M. brassicae* neonates fed *M. brassicae* NPV.
Key: as Figure 7.6.

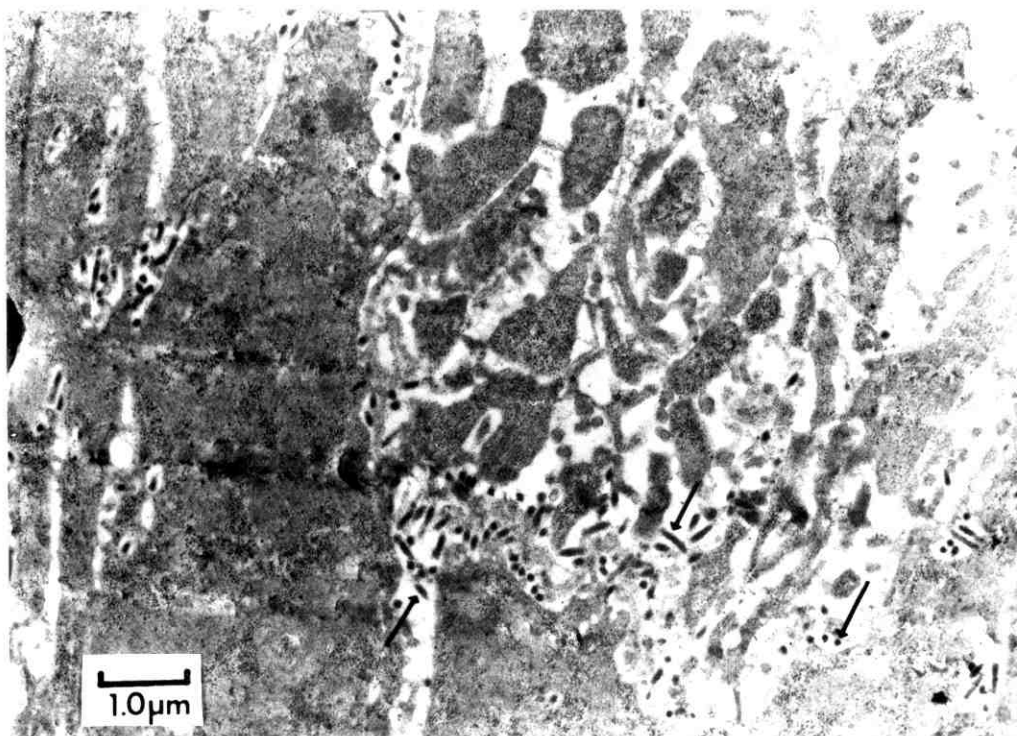


Figure 7.9. Haemocoel of *P. rapae* neonate infected with *P. brassicae* GV. (arrows indicate membrane-budded virus particles).

that uptake may be a limiting factor in infection. In contrast, when P. rapae larvae were infected with P. brassicae GV, very few virus particles were found in the lumen and nucleocapsids were seen in microvilli (not shown*). In this species the peritrophic membrane was not clearly visible (Figure 7.4). Further work would be necessary to confirm that there is a difference in uptake between these two virus types.

It was interesting that 20 hours after infection, large quantities of free nucleocapsids were evident in the haemocoel of P. rapae larva infected with P. brassicae GV, but not in the homologous NPV infection of M. brassicae (Figure 7.9). The more rapid development of this GV infection agrees with LT50 data in Chapter 6.

(4) Discussion

The examination of in vitro dissolution, showed that P. brassicae GV I.B.'s were susceptible to alkaline dissolution at lower pH than those of the noctuid baculoviruses. Ripa (1978) also found that this virus could be rapidly dissolved in 0.1 M carbonate buffer at pH $>9 <10$.

These differences were not, however, reflected in what was found in vivo. This indicates that either the gut pH is sufficient to dissolve all the I.B. types, or that ~~other~~ mechanisms, such as enzyme action, are important. The virus which was most resistant to in vitro dissolution, A. segetum (E) NPV, dissolved completely in susceptible and non-susceptible larvae. While P. brassicae GV dissolved more rapidly than A. segetum (E) NPV in P. rapae, it appeared that both completely released their virus particles before reaching the midgut (Figure 7.3). It is

* These photographic negatives were accidentally destroyed before processing.

concluded that virus particle release is not a limiting factor in baculovirus infection.

The brief examination of the fate of released virus particles, by electron microscopy, suggested that virus uptake may be an important limitation to infection. This certainly appeared to be the case with the homologous NFV infection of M. brassicae NPV. Here, the peritrophic membrane seemed to restrict movement of virus particles from the lumen to the microvilli. On the other hand, polyhedra penetrated this membrane on some occasions. It was not possible to confirm that the uptake of P. brassicae GV virus particles was more efficient. Some results presented by Ripa (1978) could be reinterpreted to this end. He fed P. brassicae GV to fifth instar P. brassicae, L. oleracea and Spodoptera littoralis larvae, and then bioassayed the gut contents in P. brassicae. The virus mortality caused by midgut contents from P. brassicae was much lower (4%) than that in the case of the other species (41% and 74% respectively). He ascribed this to differences in virus inactivation. However, it is also possible that the virus had been taken up in P. brassicae to a greater extent than in the other, non-susceptible, species.

If there is a difference in uptake between infections of susceptible hosts by P. brassicae GV and by M. brassicae NPV, this would explain why very few nucleocapsids of P. brassicae GV are required to infect P. rapae compared with any of the noctuid baculovirus infections. The wider significance of this is considered in the next Chapter.

CHAPTER 8 GENERAL DISCUSSION

<u>CONTENTS</u>	<u>PAGE</u>
(1) INFECTIVITY OF VIRUS PARTICLES AND NUCLEOCAPSIDS	212
(2) I.B. STRUCTURE AND INFECTIVITY	215
(3) I.B. POPULATION ECOLOGY	218
(4) PHYSIOLOGICAL ASPECTS OF INFECTION	220
(5) THE SIGNIFICANCE OF INFECTION PARAMETERS FOR MICROBIAL CONTROL OF INSECT PESTS	221

CHAPTER 8

GENERAL DISCUSSION

In this Chapter, particular emphasis is placed on the relationship between I.B. structure and the infectivity of I.B.'s and their components. A theory is developed which explains the variations in numbers of infective particles occluded by different viruses. This theory is based on results in the present study, in addition to the literature summarised in Chapter 1.

(1) Infectivity of Virus Particles and Nucleocapsids

Table 10 gives the results of LD50 tests, expressed as the numbers of virus particles (CPV infections) or nucleocapsids (baculovirus infections). These were calculated by multiplying I.B. LD50 values (Table 8) by the mean number of the appropriate component per I.B. of each virus (Table 5). The numbers of baculovirus nucleocapsids rather than virus particles were chosen, as the nucleocapsid is the smallest infective unit which is found individually packaged in an I.B. Virus particles from mNPV's contain variable numbers of nucleocapsids (Chapter 4). The significance of multiple, as opposed to single, envelopment of nucleocapsids, is discussed in Section 4.

Among baculovirus infections, LD50's expressed as numbers of nucleocapsids, were often similar, or of the same order of magnitude, for infections by alternative viruses in one host. This was true despite large variations in the numbers of nucleocapsids per I.B. in the different viruses. For example, while the LD50's of A. segetum GV and A. segetum (E) NPV for A. segetum neonates were 11,000 and 10 I.B.'s

Table 10 LD50's For Virus Infections, In Neonate Larvae, Converted To Equivalent
Numbers Of Virus Particles (CPV) And Nucleocapsids (Baculoviruses)

Virus	Host Species						
	<u>A. segetum</u>	<u>A. exclamationis</u>	<u>N. pronuba</u>	<u>M. brassicae</u>	<u>L. oleracea</u>	<u>P. gamma</u>	<u>P. rapae</u>
<u>M. brassicae</u> NPV	210,000	1,100,000	1,000	2,100	20,000	350	—
<u>A. segetum</u> (E) NPV	7,100	280,000	200,000	—	—	—	—
<u>A. segetum</u> (F) NPV*	8,900	—	—	—	—	—	—
<u>A. segetum</u> (P) NPV	76,000	—	—	—	—	—	—
<u>P. gamma</u> sNPV	—	—	—	—	—	38	—
<u>A. segetum</u> GV	11,000	1,200,000	—	—	—	—	—
<u>P. brassicae</u> GV	—	—	—	—	—	—	12
<u>P. gamma</u> CPV	—	—	7,500	25,000	—	1,900	—

Key: * While the French isolate had smaller I.B.'s (Table 3), its other I.B. characteristics were assumed to be the same as the biochemically indistinguishable English isolate (Chapter 4).

— = non-infectious or not tested (see Chapters 5, 6).

respectively (Table 8), these values corresponded to 11,000 and 7,100 nucleocapsids. Despite the higher LD50 of A. segetum (F) NPV I.B.'s than A. segetum (E) NPV I.B.'s in the homologous host (Table 8), this also corresponded to a similar number (8,900) of nucleocapsids (Table 10). The infections of L. oleracea neonates with L. oleracea GV and M. brassicae NPV also had similar nucleocapsid LD50's, of about 10,000 and 20,000 respectively (Chapter 6). The three infections of A. exclamationis had the highest nucleocapsid LD50's of those examined (Table 10), again with little variation between these values. On the other hand, the two infections of P. gamma neonates had the lowest nucleocapsid LD50's of the noctuid infections, at 350 with M. brassicae NPV and 38 with P. gamma sNPV.

Not all the baculovirus infections fitted this pattern of similar nucleocapsid LD50's in any one host. For example, LD50's for M. brassicae NPV and A. segetum (P) NPV in A. segetum neonates were much higher than those for homologous viruses in this host. (Table 10; note comments on A. segetum (F) NPV in Chapter 6, Section 4.) There was also a 200 fold difference in nucleocapsid LD50's for the two baculovirus infections of N. pronuba (Table 10).

These results are summarised as follows. The homologous virus, where tested, was the most infectious for each host. Even so, homologous infections often had high nucleocapsid LD50's for neonates, this value varying in each host species. Heterologous infections had similar or much lower infectivity than the homologous infection.

The following conclusion are drawn:

(a) Each host species appears to have a generalised resistance to baculovirus infection, the level of which varies from species to species,

which limits the maximum infectivity of any virus in that species.

This resistance is low in P. rapae neonates, moderate with P. gamma, high with A. segetum and extremely high with A. exclamationis.

(b) Superimposed on this generalised resistance are differences in virus specificity. Thus, while P. rapae neonates were highly susceptible to P. brassicae GV, they were not at all susceptible to the other viruses. Relative specificities were seen in the noctuid infections, where in all cases hosts were more susceptible to homologous than non-homologous infections.

Infections with P. gamma CPV supported the idea that larvae of different species exhibited different degrees of a generalised resistance to virus infection. The ratios between the P. gamma CPV LD50's were similar to those between M. brassicae NPV LD50's in the same hosts (Table 10). P. gamma neonates were the most susceptible in both cases, with the LD50's in N. pronuba and M. brassicae being 3.9 and 13.2 fold greater, respectively, for P. gamma CPV, or 2.9 and 6.0 fold greater for M. brassicae NPV. This phenomenon was also observed by Watanabe (1966(b)), who reported a significant relationship between the susceptibility to CPV and NPV infection of different B. mori strains.

(2) I.B. Structure and Infectivity

The virus isolates exhibited large differences in the mean number of infectious units occluded within I.B.'s (Table 5). While one role of the I.B. may be protection (Chapter 1, Sections 1, 7(b)), this does not explain the observed differences in packaging of infectious units.

LD50's for neonate larvae of the homologous hosts were less than 25 I.B.'s in infections by 5 of the 6 polyhedral virus isolates (Table 8).

The exception, A. segetum (P) NPV, was discussed in Chapter 6. This pattern, of low LD50's for homologous neonate larvae, is generally supported by other studies (Chapter 1, Section 6). It appears that polyhedral I.B.'s contain sufficient infectious units so that very few I.B.'s are required to infect homologous neonates. If this applies, then the mean size of I.B.'s from each isolate may be controlled by the number of infectious units required to infect the homologous host. This number may itself be governed by the level of resistance to infection of the host species (Section 1). The relationship between I.B. size and host resistance clearly applied to A. segetum (E) NPV and P. gamma sNPV. These had the largest and smallest I.B.'s of the NPV's examined, A. segetum (E) NPV I.B.'s containing 20 fold more nucleocapsids than P. gamma sNPV I.B.'s. In parallel, A. segetum neonate larvae were less susceptible to virus infection than P. gamma neonates (Section 1).

GV's by definition, contain a single infectious unit in each I.B. Few P. brassicae GV I.B.'s were required to infect P. rapae neonates, which are probably the natural hosts of this virus (Chapter 1, Section 6). This fits the pattern shown by polyhedral viruses in their homologous hosts (above). In contrast the LD50's of A. segetum GV and L. oleracea GV, in neonates of their respective homologous hosts, were high (about 10^4 I.B.'s). This would be expected as these hosts are relatively resistant to virus infection (Section 1). There are several other examples in the literature of the high I.B. LD50's of GV's in their homologous noctuid hosts, relative to the homologous NPV infection (Chapter 1, Section 6). In fact, it appears that where NPV infections occur naturally in a host, any homologous GV has low infectivity in terms of I.B. numbers. This would be expected if the nucleocapsid LD50's of the NPV and GV were similar, as observed with A. segetum (E) NPV and A. segetum GV in the

homologous host. The corollary of this is that where GV infections have low LD50's for neonates of the homologous host, no natural NPV infections are found in these hosts (e.g. P. brassicae GV, C. pomonella GV; Chapter 1, Section 6). These observations point to a possible selective advantage for polyhedral viruses (i.e. ones containing many infectious units per I.B.) in situations where nucleocapsid infectivity is low. The nature of this advantage is discussed in Sections 3 and 4.

The GV's with high I.B. LD50's for homologous neonates, such as A. segetum GV and L. oleracea GV, seem anomalous in retaining a non-polyhedral I.B. structure. In fact, many noctuid GV's sometimes have multiply enveloped virus particles, or capsules containing several virus particles (Chapter 1, Section 3; Chapter 4). This may indicate a tendency to 'develop' polyhedral characteristics. However, although the GV subgroup may have originally evolved from an ancestral sNPV (Rohrmann et al., 1981), it is now somewhat distinct from the NPV subgroup both biochemically and biologically in terms of the infection pathway (Chapter 1, Sections 2 and 4; Chapter 3). It is possible, therefore that GV's are constrained by structural or genetic limitations from 'regression' to large NPV-like I.B.'s.

The relationship between I.B. structure and infectivity, which has been discussed in Sections 1 and 2, is summarised in the following theory. Occluded virus infections of lepidopterous larvae usually have I.B.'s which contain sufficient infectious particles to compensate for the homologous host's generalised resistance to virus infection. This results in LD50's for homologous neonates which are low in terms of numbers of I.B.'s. Where the host is highly susceptible to infection, GV I.B.'s are the most economic form for packaging infectious units.

In this situation the polyhedral form would be unnecessary and, perhaps, disadvantageous (Section 3). GV's do exist alongside NPV's in very resistant hosts (such as A. segetum), where the GV I.B. LD50 is very high. This shows that the polyhedral form is not essential for survival in this type of host.

The significance of different I.B. designs is discussed below in the contexts of virus ecology (Section 3) and the infection process (Section 4).

(3) I.B. Population Ecology

Occluded viruses are found in those (holometabolous) host insects where the susceptible stages are not continuously available (Chapter 1, Section 1). Survival of virus involves successful infection of the next generation by the ingestion of infectious units, as vertical transmission of infection is probably infrequent (Chapter 1, Section 7). Between larval generations, the virus present in the environment is inactivated and spread (Chapter 1, Section 7). Where many infectious units are occluded in a polyhedron, their environmental separation is prevented and they remain as a 'package' for consumption by an individual host. This situation would be advantageous if simultaneous ingestion of many infectious units by one host were more likely to result in a successful infection than ingestion of one, or a few, units by several hosts (this is considered in Section 4). The effect of packaging many infectious units in each I.B., is to give the distribution of viral inoculum a minimum spatial heterogeneity. This will have most impact on neonate larvae which only require a few I.B.'s for infection. Older larvae are often less susceptible and require hundreds or thousands of I.B.'s

(Chapter 1, Section 6). In the latter case the clumping of infectious units into I.B.'s would not seem to increase the possibility of infection.

The infectious units produced during infection might be packaged in a few large I.B.'s or many small ones. As described above, the selection pressure operating on I.B. size may be controlled by the absolute infectivity of the infectious units in the homologous host. I.B.'s which are too small in this sense will be less able to successfully initiate infections. On the other hand, if the I.B.'s are too large, fewer will be produced and fewer infections will occur. Absolute infectivity can change following adaptations by the virus to increase it, or by the host to decrease it, and it is anticipated that the number of infectious units per I.B. will evolve in parallel.

When the infection of P. rapae with P. brassicae GV is compared with the noctuid baculovirus infections, it appears to be much more efficient. The numbers of nucleocapsids produced in the infections are probably similar (Chapter 6) although accurate estimates of GV I.B. production remain to be done. P. rapae is so susceptible to infection that the potential number of larvae which can be infected is much greater. It is interesting to speculate that such an efficient infection has eliminated other natural virus infections of P. rapae (such as NPV infections). In contrast, the lower efficiency of the noctuid baculoviruses may allow the coexistence of several different virus infections in a population of one host species. There is evidence in the literature that the mNPV's have a wider host range than the GV's or sNPV's (Chapter 1, Section 5), and this was partly supported by the present study (Chapter 5). The potential to multiply in a variety of different species may partly compensate for relatively inefficient infection of each.

The significance for control, of the varying parameters of multiplication efficiency of the different viruses, is considered in Section 5.

(4) Physiological Aspects of Infection

The existence of polyhedral I.B.'s suggests that there can be a co-operative interaction between the infectious units enclosed within. There is little evidence for any other advantages of polyhedra, such as increased environmental protection of the infectious units, although further studies of the mechanisms of I.B. degradation in the environment are necessary to confirm this. The fact that I.B.'s release their infectious units rapidly and non-specifically at ingestion (Chapter 7), also precludes differential dissolution of the various I.B. types as an important factor in infection. The relationship between the number of infectious units packaged, and their infectivity in the natural host (Section 2), leads to the conclusion that the multiple packaging of these units, as such, is the major function of polyhedral I.B.'s. If the infectious units do co-operate during infection, how they do this is open to speculation. Saturation of a host defence mechanism would be the type of interaction expected. The existence of multiply enveloped nucleocapsids in mNFV's is interesting in this context. While several singly enveloped nucleocapsids will, presumably, infect separate cells, if the same number are multiply enveloped they will initially infect only one cell (Chapter 1, Section 4). This suggests that multiply enveloped nucleocapsids may have developed in order to increase cellular infection potential, again by a co-operative effect. Where nucleocapsids pass straight through the gut epithelial cells and bud into the haemolymph, the difference between multiply and singly enveloped forms is lost (Granados

and Lawler, 1981). Therefore, any function of multiple-envelopment presumably lies at the early stages of gut epithelial cell infection. It appears that the mNFV virus particle has more resources tied up in the infection of each cell than the sNFV virus particle, in the same way that the NFV I.B. has more resources involved in infecting each larva than the GV I.B. Further studies of the early stages of infection are required to test the validity of this argument. It would be particularly valuable to know how many gut cells must be initially infected in different hosts to cause an infection leading to death, and how relatively infective multiply and singly enveloped nucleocapsids are in vivo (as opposed to in vitro - Volkmann et al. (1976)).

In Chapter 7 it was suggested that virus uptake may be an important limitation to infection, with the peritrophic membrane a major barrier. This is also supported by data in the literature (Chapter 1, Section 4). However, this is not the type of defence mechanism which could be overcome by simultaneous ingestion of several infectious units. Probably, a variety of other different mechanisms are also involved in defence against infection.

(5) The Significance of Infection Parameters for Microbial Control of Insect Pests

The LD50 and LT50 of virus infections are characteristics of fundamental importance in the choice of microbial control agents. In many control situations, viruses are used in much the same way as chemical pesticides. Where several viruses are available for use against a particular pest, the LD50's, in terms of numbers of infectious units, will indicate the dosage rates required. Where the costs of

producing different viruses are similar, the LD50's will, therefore, give the relative costs of spraying these viruses. Where very efficient virus infections are involved, such as P. brassicae GV in P. rapae, the costs of control should be lower than with the relatively inefficient infections of many noctuids (Section 3).

The LT50 can provide an indication of the period during which damage can be caused by the pests, after spraying and before death. In baculovirus infections, insects usually continue feeding until shortly before death, while with CPV's, feeding often stops shortly after infection (Chapter 1, Section 4). In the latter case the LT50, however long (Table 9) may be unimportant.

The present study suggests that the naturally occurring viruses found in pest species will be the most effective agents for control of that species. Moreover, it would not be expected that a much more infectious virus would be found. In the control of A. segetum, for example, A. segetum (E) NFV and A. segetum GV should be about equally effective, assuming no difference in persistence in the field. A. segetum GV has already been used in experimental control of this pest (Zethner, 1980(b)). Similarly, L. oleracea was equally susceptible to M. brassicae NFV and L. oleracea GV nucleocapsids (Section 1) and these viruses would, on this evidence, be equally effective for infection of this species. However, the wider host range of M. brassicae NFV may make it the more suitable in some situations where the two species occur together.

Where a virus has a wide host range it may attract greater commercial interest. In the present study P. gamma CPV would fulfill this criterion. Wider host range may indicate potential ecological hazards, however, (Payne, 1982), and further studies are required before CPV's can be considered safe in this sense.

SUMMARY

(1) The following virus isolates were obtained and passaged in their homologous hosts: M. brassicae NPV; A. segetum (E) NPV; A. segetum (F) NPV; A. segetum (P) NPV; P. gamma sNPV; A. segetum GV; P. brassicae GV; P. gamma CPV.

(2) These viruses were partly characterised, where necessary, as a preliminary to an examination of their biological properties. When examined by electrophoresis of DNA restriction endonuclease digests, and polyacrylamide gel electrophoresis of polypeptides, A. segetum (E) NPV and A. segetum (F) NPV appeared identical. However, A. segetum (P) NPV and M. brassicae NPV were clearly distinct viruses by these criteria. Analysis of P. gamma NPV samples, by restriction enzyme analysis, allowed isolation of the sNPV used in later studies, and two mNPV variants with similar DNA profiles to an NPV from A. californica. The A. segetum NPV NPV isolates, A. segetum GV and P. gamma sNPV, had not previously been biochemically analysed, and did not appear closely related to each other or to other baculoviruses.

The RNA segment profile of P. gamma CPV was identical to the published profile of a CPV from S. eximpta.

(3) Serological methods were not suitable for unequivocal virus identification due to cross reactions between antisera and heterologous antigens. The indirect ELISA method was used to examine serological relationships between the baculoviruses. This analysis showed that the morphological subgroupings of mNPV's, sNPV's and GV's were mirrored by antigenic differences.

(4) The structure of viral I.B.'s was examined, and methods devised to calculate the numbers of nucleocapsids and virus particles occluded by the different isolates. A. segetum (E) NPV had both the largest I.B.'s, and the highest numbers of nucleocapsids in each virus particle, of the baculoviruses. P. gamma sNPV had the smallest I.B.'s of the NPV's, but the highest density of virus particles. The GV I.B.'s contained single virus particles, although some A. segetum GV virus particles enveloped several nucleocapsids. P. gamma CPV had a much higher density of virus particles than the baculoviruses.

(5) The host ranges of the viruses were assayed in neonate larvae of six noctuid species from three subfamilies, and P. rapae. When infection occurred the progeny virus was identified by repeating the cross-transmission in older larvae. M. brassicae NPV had the widest host range of the baculoviruses, infecting all the noctuids. A. segetum (E) NPV and A. segetum GV infected only Agrotis spp. and N. pronuba. A. segetum (F) NPV appeared to replicate to a limited extent in M. brassicae. P. gamma sNPV infected only the homologous host and P. brassicae GV only P. rapae. P. gamma CPV infected P. rapae as well as two noctuids tested, and therefore had the widest taxonomic range of hosts.

(6) Examination of infectivity showed that most of the viruses had a LD50 of several I.B.'s for neonates of the homologous host, often being less infective for heterologous hosts. The LT50's for virus infections varied with virus type, being longest with P. gamma CPV and shortest with P. gamma sNPV and P. brassicae GV. M. brassicae NPV infections, in particular, showed a correlation between LD50 and LT50 in alternative hosts. A. segetum GV had a high I.B. LD50 for the homologous host, in contrast to most other viruses. The high I.B. LD50 for noctuid GV's, as

compared with NPV's, was confirmed by a preliminary assay of L. oleracea GV in the homologous host, and by several published examples. When LD50's were considered as numbers of infectious units, however, differences between LD50's for different I.B. types were often largely removed. These results were explained by the theory that each host has a level of general resistance to infection by any virus. I.B.'s of the homologous polyhedral virus contain sufficient infectious units to overcome this resistance.

(7) Studies of I.B. dissolution suggested that, while the dissolution rate of different viruses varies in vitro, this does not limit release of infectious units in vivo. However, limitations to virus uptake by the gut epithelium, may be one source of differences in susceptibility to infection.

APPENDIX 1 A COMPARISON OF THE ELECTROPHORETIC MOBILITIES OF
VIRAL ds RNA SEGMENTS AND ds DNA RESTRICTION
ENDONUCLEASE FRAGMENTS, ON THREE GEL SYSTEMS

<u>CONTENTS</u>	<u>PAGE</u>
(1) CPV AND <u>P. CHRYSOGENUM</u> VIRUS ds RNA SEGMENTS	227
(2) BACULOVIRUS AND λ PHAGE DNA FRAGMENTS	233
(3) EFFECT OF CHANGES IN GEL BUFFER SYSTEMS	234
(4) DISCUSSION	234

APPENDIX 1

A COMPARISON OF THE ELECTROPHORETIC MOBILITIES OF VIRAL ds RNA SEGMENTS AND ds DNA RESTRICTION ENDONUCLEASE FRAGMENTS, ON THREE GEL SYSTEMS

Three gel systems, 1% agarose, 3% polyacrylamide and 10% discontinuous polyacrylamide (Chapter 2) were compared for the analysis of double stranded genome viruses. Genome segments were purified from CPV polyhedra as described in Chapter 2. The preparations of Bombyx mori and Inachis io CPV's were provided by Dr. C. Payne (G.C.R.I.), while N. promuba CPV originated in a cross-transmission test in which N. promuba were orally inoculated with A. segetum (E) NPV (Chapter 5). Purified ds RNA from the segmented genome virus of Penicillium chrysogenum was obtained from Dr. R. Barton (G.C.R.I.), and examined in comparison with the CPV genome segments. Baculovirus and λ phage ds DNA's were digested with the restriction endonuclease EcoRI or Hind III (Chapter 2), before electrophoresis in parallel with the RNA viruses.

The results of this investigation are illustrated in Figures A.1 to A.4

(1) CPV and P. chrysogenum Virus ds RNA Segments

On agarose gels the low molecular weight CPV segments ($<10^6$) were often faint or absent. This is probably attributable to relatively high diffusion rates within the low concentration gel. Figure A.1 illustrates this phenomenon with RNA's of B. mori CPV ('Type 1' using the classification of Payne and Rivers, 1976), I. io CPV (Type 2) and N. promuba CPV (Type 7). The RNA samples used on this occasion were

purified from polyhedral preparations by the cold phenol method usually adopted for DNA purification (Chapter 2). The faint high molecular weight bands, about 2 cm below the sample wells on the I. io CPV and N. promuba CPV tracks, were not found when RNA preparations were used which had been purified by hot phenol, which eliminates DNA (Scherrer, 1969). When the cold phenol-extracted RNA's were digested with EcoRI, these bands disappeared. In the case of I. io CPV, bands corresponding to the M. brassicae NPV DNA EcoRI digest were found, together with the I. io CPV RNA bands (Figure A.1). This I. io CPV preparation had been grown in M. brassicae, where a contaminant NPV infection probably occurred. The lack of DNA fragment bands in the N. promuba CPV digest may indicate insufficient DNA contamination for resolution of these bands.

CPV ds RNA segment profiles on the other gel systems were similar to those on agarose gels. All 10 bands were clearly resolved on 3% and 10% polyacrylamide gels, being most sharply-defined on the higher concentration gel (Figures A.3, A.4). However, some of the segment bands varied in relative positions on the different gels. This was particularly true of the bands at molecular weights greater than 2×10^6 (calculated using the B. mori CPV segment molecular weights given by Fujii-Kawata et al. (1970) as standards). For example, only three of the highest molecular weight segments of I. io CPV were resolved on 3% polyacrylamide (Figure A.3), compared with four on agarose and 10% polyacrylamide (Figures A.1 and A.4). In addition, one of the segments had a much lower mobility, compared with the B. mori segments, on 10% polyacrylamide than the alternative systems. The P. chrysogenum virus RNA also exhibited different patterns on the three gel systems, apparently containing three segments on 3% polyacrylamide (Figure A.3), but four segments on the other systems (Figures A.2, A.4). Previous studies considered that this

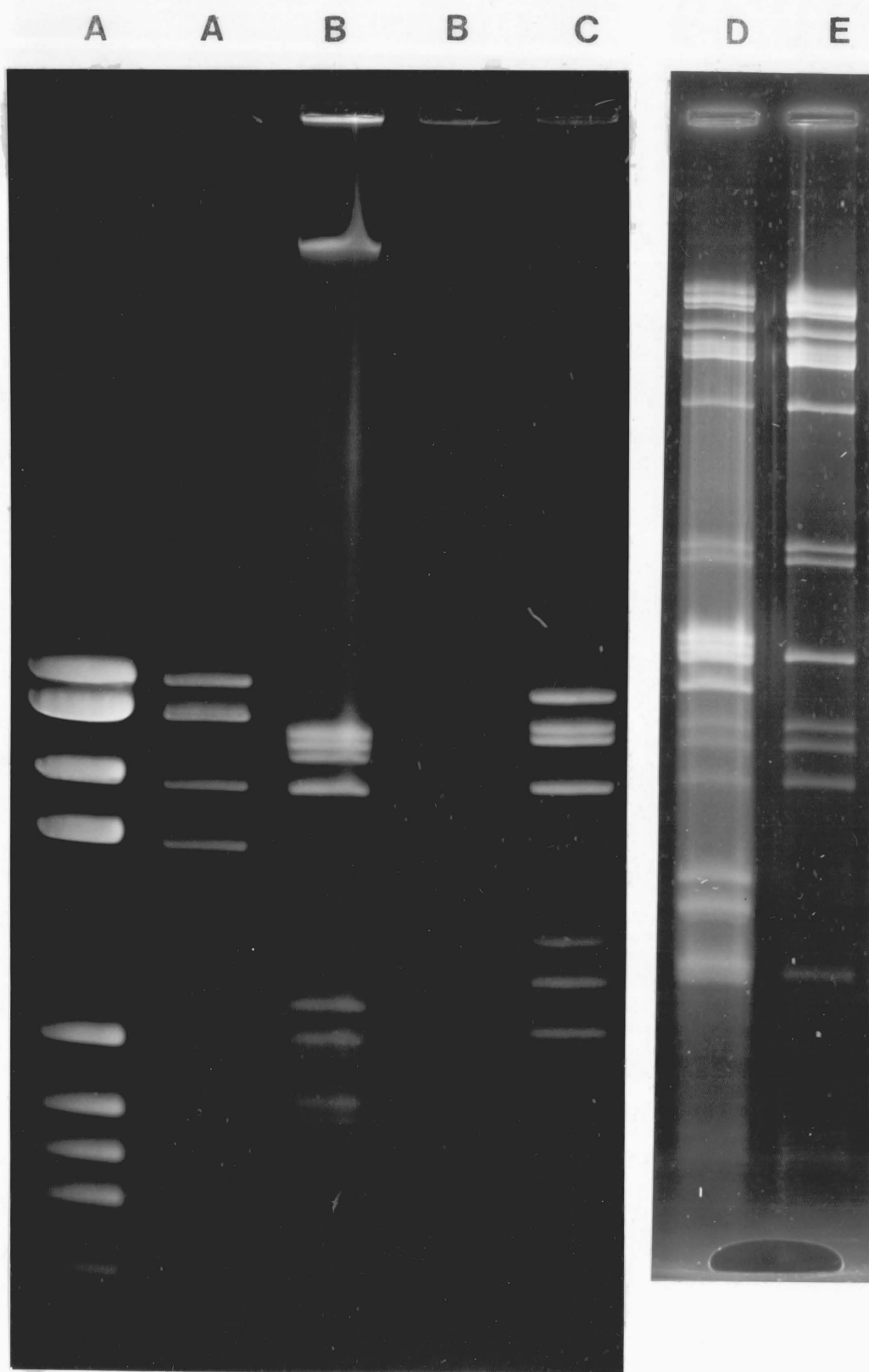


Figure A.1. Electrophoresis of nucleic acids on 1% agarose.

Key: A: *B. mori* CPV RNA. B: *I. io* CPV RNA (+DNA).
 C: *N. pronuba* CPV RNA. D: *I. io* CPV RNA, +DNA
 digested with EcoRI. E: *M. brassicae* NPV DNA digested
 with EcoRI. (A, B, C run separately from D and E).

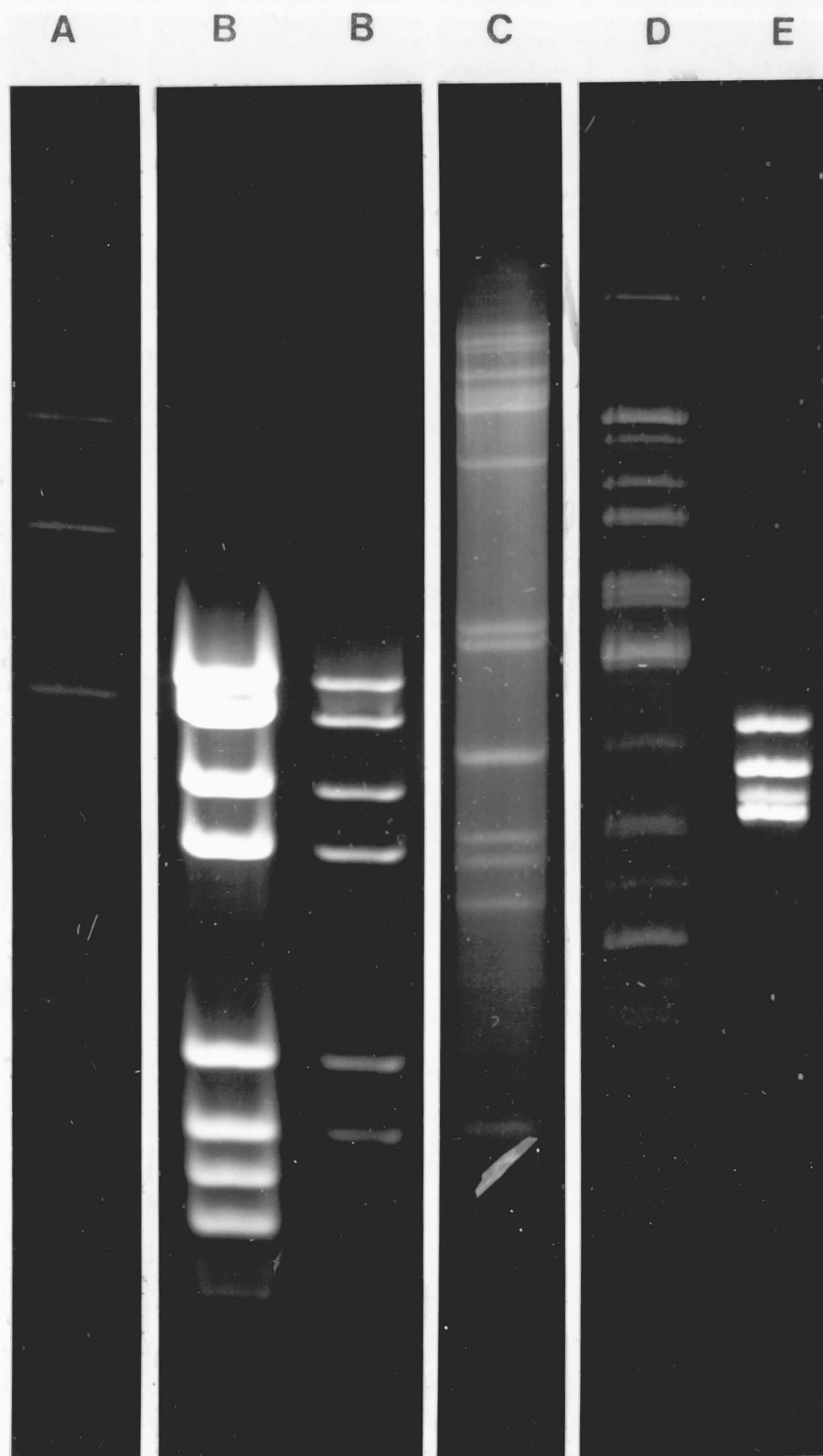


Figure A.2. Electrophoresis of nucleic acids on 1% agarose.

Key: A: λ DNA, Hind III digest. B: *B. mori* CPV RNA.
 C: *M. brassicae* NPV DNA digested with EcoRI.
 D: *A. segetum* (E) NPV DNA digested with EcoRI.
 E: *P. chrysogenum* virus RNA. (all samples run on one gel, intervening sample profiles removed).

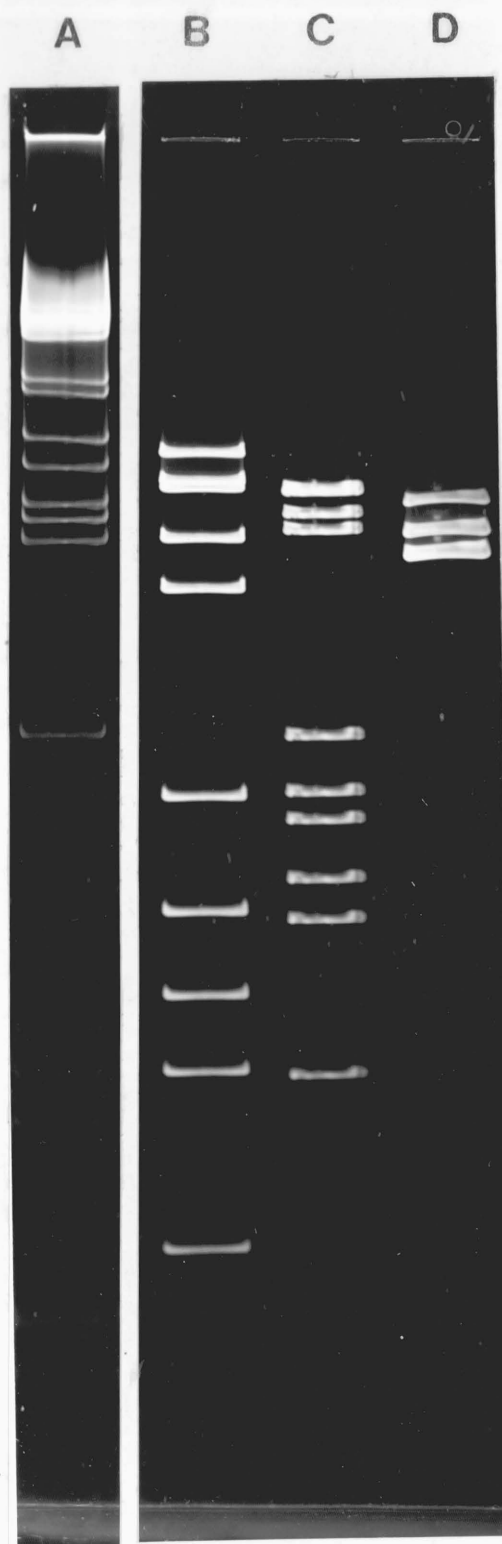


Figure A.3. Electrophoresis of nucleic acids on 3% polyacrylamide.

Key: A: *M brassicae* NPV DNA digested with EcoRI.
 B: *B. mori* CPV RNA. C; *I. io* CPV RNA.
 D: *P. chrysogenum* virus RNA. (all samples run on one gel, intervening sample profiles removed).

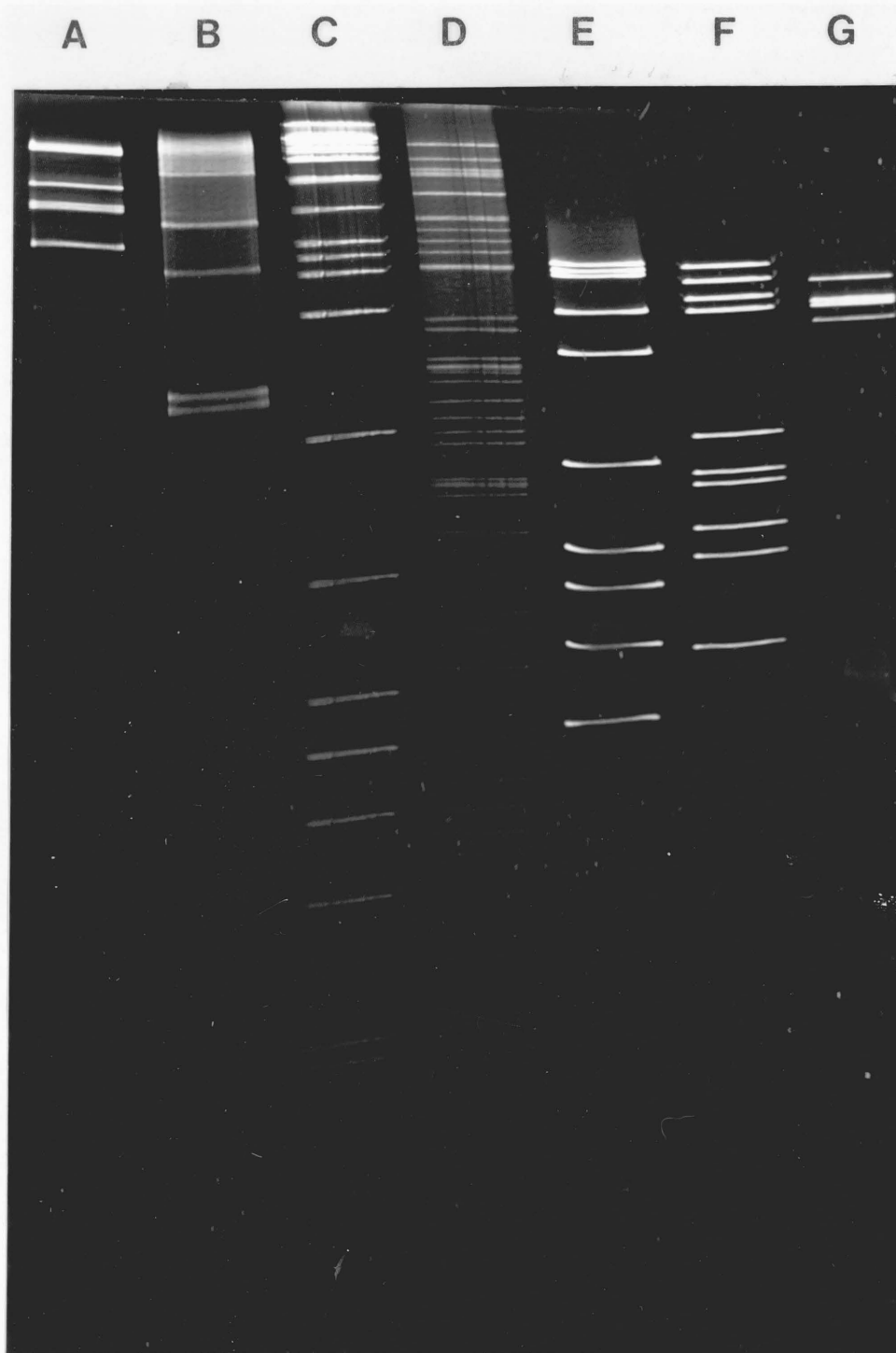


Figure A.4. Electrophoresis of nucleic acids on 10% discontinuous polyacrylamide.

Key:

- A: λ DNA digested with EcoRI.
- B: λ DNA digested with Hind III.
- C: *M. brassicae* NPV DNA digested with EcoRI.
- D: *A. segetum* (E) NPV DNA digested with EcoRI.
- E: *B. mori* CPV RNA.
- F: *I. io* CPV RNA.
- G: *P. chrysogenum* virus RNA

virus contained three RNA segments when electrophoresed in 2-3% polyacrylamide gels (Nash et al., 1973; Wood and Bozarth, 1972; Buck and Girvan, 1977). The present study shows that an additional segment could be resolved, at least in this particular preparation, by electrophoresis at much higher or lower gel concentrations.

(2) Baculovirus and λ Phage DNA Fragments

Restriction endonuclease fragments of ds DNA samples showed different patterns on the three gel systems, as had the ds RNA segments. For example, the M. brassicae NPV DNA EcoRI digest profile is shown in Figures A.2-A.4. While it was possible to identify the equivalent fragments of the λ phage DNA Hind III digest on the three gels (Figures A.2, A.4, 3% polyacrylamide profile not shown), the profile of the A. segetum (E) NPV DNA EcoRI digest had too many bands for this to be done confidently (Figures A.2, A.4). Resolution was greatest on 10% polyacrylamide gels. Many of the smaller fragments (which disappeared on agarose gels due to high mobility or diffusion) were resolved.

When the mobilities of λ phage DNA Hind III fragments as well as B. mori CPV genome segments were compared on a molecular weight basis, they were similar on all three gel systems. As gel concentration increased, however, the DNA mobility decreased relative to that of RNA, which can be seen by comparing the relative positions of these two digest profiles in Figures A.2 and A.4. The positions of the λ Hind III fragments on 3% polyacrylamide (not shown) were intermediate between those on the other two systems.

(3) Effect of Changes in Gel Buffer Systems

When the discontinuous 3% upper gel/10% lower gel system of Laemmli (Chapter 2) was replaced by a single 10% polyacrylamide gel with the electrode buffer used for agarose gels (Chapter 2), the profiles for nucleic acid segments and fragments were unaltered. Similarly, the profiles on 3% gels were the same using the agarose buffer system or that of Payne and Tinsley (1974) (Chapter 2). When nucleic acid samples were prepared in the different sample buffers of Laemmli (1970), Loening (1967) or the DNA digest buffer (Chapter 2), the profiles produced were the same on the three systems.

It was concluded that the observed variations in migration of nucleic acid segments and fragments on the three gel systems was due to differences in the gel concentrations, rather than different buffer conditions.

(4) Discussion

Gels of a higher concentration produced sharper bands and resulted in reduced mobility differences between high and low molecular weight nucleic acid segments or fragments. The latter phenomenon was also noted by Bozarth and Harley (1976) using polyacrylamide gels. The gel concentration also affected the pattern of bands for each virus profile, perhaps caused by variations in the structural characteristics of the nucleic acid segments or fragments (Helling et al., 1974). In this case higher concentration gels would be expected to give less accurate molecular weight estimates. While CPV's have not previously been analysed on 10% polyacrylamide or agarose gels, Sharpe et al. (1978) found that reovirus segments migrated differently on 5% and 10% acrylamide gels.

High concentration polyacrylamide gels have potential for very precise examination and comparison of baculovirus DNA restriction endonuclease digests and CPV genome segments. Agarose gels, on the other hand, may give better molecular weight estimates, because at lower gel concentrations differences in the secondary structures of ds nucleic acids will have less effect on mobility. These gels were also useful, in the present study, for investigation of baculovirus/CPV mixtures. Where baculovirus or CPV preparations are grown in vivo there is the possibility of contamination with other occluded viruses. A useful routine test, in these cases, is the cold phenol extraction of nucleic acid from the preparation, followed by electrophoresis on agarose gels, with and without endonuclease digestion.

APPENDIX 2

STATISTICAL APPENDIX TO CHAPTER 4

a) Calculation of Polyhedral Volume from the Mean and Variance of the Diameter

For a random variable, x , with mean Mx and variance σ_x^2 , the mean and variance of a function, $y = g(x)$, are approximated by:-

$$\text{mean, } My = g(Mx) + \frac{g''(Mx)}{2} \sigma_x^2$$

$$\text{variance, } \sigma_y^2 = (g'(Mx))^2 \sigma_x^2$$

where g' and g'' denote the first and second derivatives, respectively (Kendall and Stuart, 1958). If the random variable is the radius of a polyhedron, r , with mean $\bar{r}$ and variance σ_r^2 , then the mean and variance of the volume of a polyhedron are given by:

$$\text{mean, } \bar{V} = \frac{4}{3}\pi(\bar{r}^3 + 3\bar{r}\sigma_r^2)$$

$$\text{variance, } \sigma_V^2 = 16\pi^2\bar{r}^4\sigma_r^2$$

The standard error of the mean volume, $\sigma_{\bar{V}}$ is given by:

$$\sigma_{\bar{V}} = \frac{\sigma_V}{\sqrt{n}}$$

where n = number of polyhedra measured.

b) Calculation of the concentration of virus particles within I.B.'s from the Number Seen in Thin Section

The probability of sectioning a virus particle is proportional to its length perpendicular to the plane of the section, l , added to the

section thickness, t . Therefore the number of virus particles seen in a thin section is the mean number lying in the volume delineated by the area of the section multiplied by $(1 + t)$. This is a mean value rather than the absolute value, as most virus particles seen in the section will lie partly outside the limits $\frac{1}{2}l$ above and below the section, while many particles which are not sectioned lie partly inside these limits.

$(1 + t)$ is the 'sample thickness', s . The value of l is dependent on the virus particle dimensions. For the spherical virus particles of a CPV, l is equal to the diameter of the sphere, d ,

$$\text{therefore } \underline{s = d + t}$$

For rod-shaped virus particles, such as baculovirus virus particles, random orientation of the virus particles is assumed (Chapter 1, Section 3). In the case of a theoretical rod-shaped virus particle with infinitesimal width, the tips of the particle describe a sphere, for all possible orientations, with radius, r , equal to half the virus particle length. The mean thickness of this sphere is equal to the mean length perpendicular to any one plane, of the virus particle,

$$\text{therefore } l = \frac{\frac{4}{3}\pi r^3}{\pi r^2} = \frac{4}{3}r$$

For a virus particle with width, w , this has an additive effect on the mean length perpendicular to the section plane:

$$l = \frac{2}{3}w + \frac{4}{3}r$$

$$\text{therefore } \underline{s = \frac{2}{3}w + \frac{4}{3}r + t}$$

where r is half the true virus particle length,

w is the virus particle width.

When baculovirus particles were counted in thin sections, the presence of the densely stained nucleocapsids was used as the criterion for presence of virus particles. The nucleocapsid dimensions are, therefore, the relevant ones for calculation of sample thickness, s . Nucleocapsids which were multiply enveloped had combined maximum widths of approximately 2x the nucleocapsid width for virus particles with 2 or 3 nucleocapsids, 3x for those with 4, 5 or 6 nucleocapsids and 4x for those with 7-10 nucleocapsids (Figures 4.7, 4.8, 4.9). The dimension perpendicular to the sectioning plane, of each class of virus particle width was calculated separately. The mean of these was then calculated, taking into account the proportion of each size class present (Table 4). The dimensions of baculovirus nucleocapsids used for calculations were based on the estimates in Section 2 (length = 275 nm; width = 32.5 nm). The value given by Martignoni (pers. comm.) of 65 nm was used for P. gamma CPV virus particle diameter. The thin section thickness was 80 nm.

Values for sample thickness, for each of the four polyhedral isolates examined by thin section, are given in Chapter 4, Section 4(b).

c) Calculation of the Number of Virus Particles and Nucleocapsids per I.B.

The method of approximating means and variances of a function of a random variable (Section (a)), can be extended to several random variables (Kendall and Stuart, 1958). It is assumed that the I.B. volume, V , number of virus particles per unit volume, p , and number of nucleocapsids per virus particle, n , are mutually independent. The means and variances of these three distributions are, respectively, $\bar{V}$ and $6\frac{2}{V}$, $\bar{p}$ and $6\frac{2}{p}$, $\bar{n}$ and $6\frac{2}{n}$. Then the mean, $\bar{a}$, and standard error, $6\bar{a}$, of the

number of virus particles per I.B. are given by:

$$\bar{a} = \bar{V} \times \bar{p}$$

$$\begin{aligned} \sigma_{\bar{a}} &= \sqrt{\left(\frac{\partial(\bar{p}\bar{V})}{\partial \bar{p}}\right)^2 \sigma_{\bar{p}}^2 + \left(\frac{\partial(\bar{p}\bar{V})}{\partial \bar{V}}\right)^2 \sigma_{\bar{V}}^2} \\ &= \sqrt{\bar{V}^2 \sigma_{\bar{p}}^2 + \bar{p}^2 \sigma_{\bar{V}}^2} \end{aligned}$$

where $\frac{\partial}{\partial \bar{p}}$ and $\frac{\partial}{\partial \bar{V}}$ represent partial derivatives with respect to $\bar{p}$ and $\bar{V}$ respectively.

Similarly, for the mean, $\bar{b}$, and standard error, $\sigma_{\bar{b}}$, of the number of nucleocapsids per I.B.,

$$\bar{b} = \bar{V} \times \bar{p} \times \bar{n}$$

$$\begin{aligned} \sigma_{\bar{b}} &= \sqrt{\left(\frac{\partial(\bar{V}\bar{p}\bar{n})}{\partial \bar{p}}\right)^2 \sigma_{\bar{p}}^2 + \left(\frac{\partial(\bar{V}\bar{p}\bar{n})}{\partial \bar{V}}\right)^2 \sigma_{\bar{V}}^2 + \left(\frac{\partial(\bar{V}\bar{p}\bar{n})}{\partial \bar{n}}\right)^2 \sigma_{\bar{n}}^2} \\ &= \sqrt{(\bar{V}\bar{n})^2 \sigma_{\bar{p}}^2 + (\bar{p}\bar{n})^2 \sigma_{\bar{V}}^2 + (\bar{V}\bar{p})^2 \sigma_{\bar{n}}^2} \end{aligned}$$

reference: Kendall and Stuart, 1958.

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