STUDIES OF THE SUSCEPTIBILITY OF

PIERIS BRASSICAE (L.)

TO A GRANULOSIS VIRUS.

by

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TO MY PARENTS.

ABSTRACT

The aim of the present investigation was to study the factors which influence the uptake of granulosis virus (GV) from the gut of Pieris brassicae.

Using new and established techniques the following subjects were investigated :

- The production of GV from P. brassicae, its purification and the microscopical estimation of the concentration of the capsules.
- The development of a method for measuring the pH in nine sections of the alimentary canal and of regurgitated fluid using instant freezing in liquid nitrogen and a micro glass electrode.
- The development of a colorimetric method of assessing the food consumption by individuals of different instars of P. brassicae and the effectiveness of the technique.
- The dosage- and time-mortality responses of second-, third- and fourth-instar, virus-free larvae of P. brassicae to their own GV and the comparative resistance of three other stocks obtained from the Canary Islands, France and Holland.
- The viability of GV subjected to different pHs of buffers and of gut fluid and also the loss of infectivity of GV in the alimentary canal of mature larvae of P. brassicae,

Lacanobia oleracea and *Spodoptera littoralis*.

- The dissolution and degradation of the capsules in buffers and gut fluid using the electron microscope and correlation of the observations with the studies of virus viability.
- The rôle of mid-gut pH and proteolytic enzymes on GV infection in second-instar larvae using various acidified diets, and protein inhibitors.
- The range of pHs in the mid-gut of different age groups and stocks of *P. brassicae* and its relationship to the resistance of the stocks to GV.
- The origin and formation of the peritrophic membrane mainly by microscopical sections.
- The relationship between the known increase in resistance to virus during larval development and the parallel variations in mid-gut infective area.
- The rate of passage of food through the alimentary canal of fifth-instar larvae.

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INTRODUCTION

It is necessary to study the interaction between host and pathogen to understand the potentialities of pathogenic viruses for the control of insect pests with the ultimate aim of making the pathogen as effective as possible.

In the present investigation P. brassicae and its granulosis virus were used. This model system was chosen beacuse it was already established in the laboratory (David 1978, David and Gardiner 1966a and 1966b) and because this was accidentially introduced into Chile in 1970 (Gardiner 1974) and has subsequently increased in economic importance.

The granulosis virus initially infects the mid-gut of the larvae as a result of feeding on contaminated food. For this reason several factors which were likely to influence the uptake of virus from the alimentary canal were investigated.

REVIEW OF LITERATURE

Before the relationship between a host and its virus can be understood it is necessary to study those aspects of the morphology and physiology of the host and of the virus which are likely to bear on the interaction.

Most of the investigations described in the present thesis are concerned with the uptake of a granulosis virus (GV) from the alimentary canal of larvae of the large white butterfly also called European cabbage worm, Pieris brassicae (Linnaeus) (Lepidoptera, Pieridae). The following literature review is, therefore, largely confined to publications dealing with properties of the virus, the structure and physiology of the gut and methods for studying these subjects.

1. The Host : Pieris brassicae.

The large white butterfly is a pest of holartic origin and is found in Europe, coastal regions of North Africa, Eastern Mediterranean regions and parts of Asic (Commonwealth Institute of Entomology, 1976).

Recently it has been found in the Southern part of South America. In 1971 this pest was found in central Chile (Gardiner 1974) and during the last 6 years it has spread rather slowly but steadily, and is now found some 60 to 70 kms east of the original site of infestation (Gonzalez, personal communication).

This new pest caused great concern among Chilean farmers who responded with an increased use of pesticides. It is now considered probably that the insect will eventually spread in the temperate zone of South America and very likely to other areas in the Neotropical region.

2. Rearing of P. brassicae.

The requirement for a continuous supply of homogeneous test insects especially for bioassays has been stressed by Burges and Thomson (1971) and Dulmage (1973). As is well known the homogeneity of the test insects, as well as other factors, influence the slope of the dosage-mortality regression line obtained in bioassays (eg. Dulmage 1973).

Methods for rearing uniform virus-free P. brassicae larvae on a semi-synthetic diet have been described by David and Gardiner (1966a) (See Addendum). In earlier papers David and Gardiner (1952), (1961) have also described methods of housing the adults and feeding them on artificial flowers which enable a stock to be maintained throughout the year.

3. Morphology and Physiology of the Larval Gut.

As in all Lepidopterous larvae the alimentary canal of P. brassicae consists of a more or less straight tube running from the mouth to the anus. It is divided into three sections, fore-gut, mid-gut and hind-gut (Chapman

1969). The fore-gut and the hind-gut can be regarded as unimportant in the host-virus interaction. It is in the mid-gut that the inclusion body is dissolved and the virion enters the host cells.

The mid-gut epithelium is formed by long columnar cells with microvilli lining their apical surface bordering the lumen (Wigglesworth 1965). Beside these epithelial cells, especially in Lepidoptera, there are goblet cells each of which has an internal cavity lined with microvilli, usually filled with mitochondria (Smith 1968). These goblet cells seem to be involved in the active transport of potassium ions from the haemolymph to the gut lumen (Flower and Filshie 1976). These ions provide a basis for the alkalinity maintained in the mid-gut of Lepidopterous larvae. It is interesting to point out that the goblet cells have not been found to be infected by GV (Tanada and Leutenegger 1970).

As food enters the mid-gut it is surrounded by the peritrophic membrane (Wigglesworth 1965) formed at the anterior end of the mid-gut. This membrane in P. brassicae is made up of a fibrillar network and an amorphous matrix (Peters 1969).

It has not been shown how the virions released from the inclusion bodies cross the peritrophic membrane in Lepidopterous larvae. Harrap (1970) suggests that there might be holes, and Paschke and Summers (1975) have observed large discontinuities in the peritrophic membrane of Trichoplusia ni.

They add that "viral enzymes may play a significant rôle in penetration of the peritrophic membrane". Watanabe (1971) suggests that the peritrophic membrane of B. mori is responsible for a filtering action preventing most of the ingested virus from reaching the mid-gut epithelium.

Further evidence and ideas about the function of the peritrophic membrane may be obtained from work on other pathogens and parasites. In honey bee larvae it is absent during the first 2 days of life and it has been observed that an increase in resistance to Bacillus larvae occurs after this period (Davidson 1970). With age the peritrophic membrane thickens and this may be related to the observed increase in resistance to infection (Davidson 1970). In Glossina sp the peritrophic membrane is soft and viscous at the point of formation and it is only here that is can be penetrated by Trypanosoma sp (Stohler 1961). In mosquitoes encephalitis virus did not cause infection possibly because the peritrophic membrane acted as a barrier (Stohler 1961).

The alkalinity of the mid-gut provides the conditions for dissolution of the capsule which is an essential preliminary to infection (Martignoni 1957). In the case of P. brassicae the pH of the mid-gut has been reported as 9.4 (Waterhouse 1949).

Various investigators have measured the pH in the alimentary canal of insects with either indicators (Salkeld and Friend 1958, Sinha 1959, Pandey and Srivastava 1974, Dadd 1975) or with pH meters equipped with micro-electrodes (Grayson 1951, Heimpel 1955, Burton et al.,1977).

Dadd (1970) concluded that indicators give imprecise results and cannot be used for insects with coloured gut contents. Micro-electrodes, he found, offered precision but only if observations are made rapidly to avoid changes in composition due to exposure of the gut fluid to the atmosphere.

Lecadet and Dedonder (1966) showed that two proteolytic enzymes occur in P. brassicae gut fluid with an optimum activity at pH 10.5. These enzymes may be considered as related to the trypsin-chymotrypsin group but have a wider specificity and faster action. Whether these gut enzymes play a rôle in the infection process of the virus has not yet been elucidated. (Summers 1971, Kawanishi et al., 1972) However Vago and Croissant (1959) have shown that exposure to gut fluid reduces the infectivity of B. mori nuclear polyhedrosis virus to its host. For example, after an exposure of 10 minutes the virus produced 42% mortality whereas after 60 minutes exposure it produced only 12% mortality.

4. Measurement of Food and Pathogen Consumption.

Precise bioassay techniques involves the assessment of the actual dose of pathogens ingested by each test insect.

This can be achieved by feeding a known dose per insect and checking that the entire dose is consumed (Allen and Ignoffo 1969, Magnoler 1974 and 1975, Sheppard and Stairs 1977, Boucias and Nordin 1977). An alternative way is to distribute homogeneously a known concentration of pathogens in the food with a marker which will provide an individual estimate of the dose consumed.

McGinnis and Kasting (1964) tested chromic oxide as an index compound in food uptake studies with insects and recovered between 98 and 100% of the chromic oxide. The method required each sample to be digested with a perchloric acid- sulphuric acid- sodium molybdate mixture (a somewhat hazardous process), and then treated with diphenylcarbazide before measurement of the absorption.

Rogers et al., measured the dose of Bacillus thuringiensis ingested by larvae of P. brassicae using radioactive phosphorus and indicated an error equal or less than 2% which is similar to the accuracy obtained with the chromic oxide method. Rogers et al., (1966) and (1967) using radioactive P32 (orthophosphate) and White et al., (1970), radioactive zinc-65 chloride as index compounds, determined the dose of pathogens ingested by individual larvae but gave no dosage-mortality analysis of the data.

An alternative and ingenious method for dosing specially small larvae is given by Wigley (1976) where the larvae are confined in a hole made in two perspex plates with the leaf held in between, so that they are allowed to eat a pre-determined area on which the pathogen is deposited.

5. The Virus.

The GV in P. brassicae was the first virus of this type to be discovered. Paillot (1926), who made this discovery, named it pseudograsserie because he observed that it differed from the grasserie ie. nuclear polyhedrosis virus (NPV) of the silkworms.

Bergold (1948) found that GV of Choristoneura murinana contained a rod-shaped virus surrounded by an inclusion body and he called the intact virions Viruskapseln. However, the name granulosis was later proposed by Steinhaus (1949) and has become accepted.

In the case of GV of Pieris rapae Tanada (1953) observed that the virus also consisted of an inclusion body containing a single rod-shaped virus and subsequently it has been shown that P. brassicae GV has essentially the same structure (Smith 1976).

More recently the inclusion body and the enclosed virus have been examined in greater detail. The inclusion body has been shown to consist mainly of the proteinaceous granulin (Summers and Egawa 1973) surrounded by a thin

cortical layer which is more resistant to alkali (Longworth et al., 1972). The enclosed virion (rod-shaped virus) is made up of an outer layer the envelope and the nucleocapsid (see Fig. 42, Addendum). When the nucleocapsid is disrupted with alkali the DNA and core proteins are released and a delicate tubular capsid (intimate membrane) remains (Huger 1963).

(i) Production and Purification of the Virus.

So far it has only been possible to produce GV in living insects (Reed 1971, Jacques 1974, Boucias and Nordin 1977). Usually larvae are employed (David et al., 1968) and these are dosed orally, although a method using pupae has been described, (Vago and Atger 1961).

As the insects die they are usually deep frozen until a sufficient quantity are available, then the virus is extracted by filtration and centrifugation.

David et al., (1968) purified the GV of P. brassicae by grinding the dead larvae and adding water. The material was then strained through fine muslin, filtered and further purified by centrifugation.

Longworth et al., (1972) separated GV capsules from P. brassicae larvae by centrifuging at 10,000g for 20 minutes and purified them either by three cycles of centrifugation on 10- 70% (v/v) glycerol gradients for 20 minutes at 3,500g or on 10- 60% (w/v) sucrose gradients

at 3,500g for 90 minutes.

Brown et al., (1977) infected second-instar P. brassicae larvae and harvested moribund final-instar larvae. They first suspended the virus by triturating the larvae in distilled water and filtering the suspension through muslin. Then, after a preliminary centrifugation, it was layered on 20- 70% (v/v) glycerol gradients and centrifuged at 3,500g for 15 minutes. The recovered granules were further purified by centrigugation on 30- 65% (w/w) sucrose gradients at 75,000g for 90 minutes.

(ii) Uptake of Virus from the Gut.

The uptake of virus from the gut seems to occur as virions and/or as smaller infective components, after dissolution of the capsules in the alkaline gut fluids.

The infection process has been studied for NPV and GV by Harrap and Robertson (1968), Harrap (1970), Summers (1969) and (1971) and Kawanishi et al., (1972) in different Lepidopterous hosts. These authors observed that the virus envelope appear to fuse with the microvilli of columnar cells. However, Tanada and Leutenegger (1970) and Hunter et al., (1973) suggested instead that the virions may pass between the gut columnar cells, through the intercellular spaces, and basal lamina of the gut epithelium and reach the haemocoel. Infection through smaller infective components has been put forward by Summers and Paschke (1970) and Summers and Anderson (1973).

MATERIALS AND METHODS

1. The Insect Stocks, Diet and Laboratory Conditions.

Larvae of P. brassicae was used for almost all the experiments.

The origin and handling of a virus-free stock has been described by David and Gardiner (1966b). It was maintained in a carefully isolated room and has now been free from overt virus disease for 14 years.

The P. brassicae cheiranthi type stock was derived from pupae imported from the Canary Islands in 1963. It was fed on nasturtium plants (Tropaeolum majus Linnaeus) (David and Gardiner, 1965) until December 1976 when it was transferred to standard semi-synthetic diet.

A third stock was imported from France and a fourth from Holland in 1973 (David personal communication). Both stocks were fed on standard diet, and only very occasional virus deaths occurred probably because of accidental contamination.

The standard semi-synthetic diet used in the rearing and experimental work has been described by David and Gardiner (1966a). Formalin was not added when virus was applied to the diet (David et al., 1972b).

Five different sizes of containers were used with diet for the tests, glass specimen tubes, 5.0 x 2.3 cm used to

maintain insects individually, 7.5 x 3.0 cm filled with a 5 mm layer of formalin-free diet used for virus treatment of second-instar larvae (David, Ellaby and Taylor 1971b), 7.5 x 3.5 cm filled with a 1.5 cm layer of standard diet used to keep the larvae after being dosed with virus, and one pound jam jars, filled with a 1.5 to 2 cm layer of standard diet and used to rear the larvae in large numbers. All the above containers were closed with filter paper lids and held in a horizontal position. The larvae were examined and cleaned daily and diet changed every 6 to 7 days.

Cabbage, (Brassica oleracea Linneaus variety May Express) was grown in plastic pots in a screened glasshouse. This was used in some observations to rear fourth- and fifth-instar larvae in plastic boxes 22 x 13.5 and 7.5 cm high with nylon screened lids. The cabbage leaves were changed daily. The leaves from these cabbage plants were also used in certain tests to be described later.

Larvae were maintained in constant temperature rooms at 20 ± 1°C and 60 ± 5% relative humidity with a light period of 16 hours daily provided by fluorescent tubes (David and Gardiner 1962).

2. The Virus Stock.

A stock of granulosis virus which had been used in several previous investigations eg. David et al., (1968), was used to provide the inoculum used in this investigation.

(i) Infection Procedure.

Fourth- and fifth-instar virus-free larvae were used for producing the virus. One pound jam jars containing a 2 cm thick layer of formalin-free diet were treated with a suspension of larvae which had died of granulosis virus. After the surface had dried thirty fourth- or twenty fifth-instar larvae were placed in each jar. The larvae began to die after 6 to 7 days. The dead or moribund larvae were collected daily and stored at - 20°C.

(ii) Purification of the Virus.

The dead larvae were triturated with distilled water in a glass tissue grinder, and filtered through fine Terylene gauze, which removed the large pieces of host tissue. The crude virus suspension was then further diluted with distilled water and centrifuged at 121 x g for 5 minutes in a Sorvall RC2 centrifuge at 4°C using a SS-34 rotor. The pellet, containing small pieces of host debris, and bacteria was discarded in this first step.

The supernatant was pelleted at 27,138 x g for 20 minutes and then resuspended and this process was repeated until the remaining liquid appeared colourless. The semi-purified virus capsules so obtained were then resuspended in 150 ml distilled water. Further purification was carried out on sucrose gradients. For this a continuous gradient was formed using an adaptation of the apparatus described by MaCall and Potter (1973). A diagram of this apparatus is

shown in Fig. 1. It was used to place 25 ml of sucrose gradient ranging from approximately 31 to 57% w/w sucrose in each of two 50 ml polycarbonate centrifuge tubes.

Initially 40 ml of 60% w/w sucrose solution was introduced into the graduated reservoir and 13 ml of sucrose plus 12 ml of distilled water were added to the graduated mixing chamber resulting in an approximate 31% w/w sucrose solution. By pumping air into the reservoir with the rubber Griffin pipette filler the concentrated sucrose was forced into the mixing chamber. As soon as this occurred the air pressure in the mixing chamber was gradually increased with the 20 ml syringe. This allowed sucrose solution of progressively increasing concentration to flow into the bottoms of 2 centrifuge tubes via the two silicone rubber tubes of equal length. Both air pressures were adjusted so that by the time the reservoir was empty only 14 ml of about 57% sucrose were left in the mixing chamber.

At this point the filling of the first two tubes was stopped. Two new centrifuge tubes were started. For this 11 ml of distilled water were added to the mixing chamber (bringing the concentration back to 31%) and 40 ml of 60% sucrose to the reservoir.

For the purification of the virus each density gradient tube was overlayed with 4 ml of semi-purified virus suspension and centrifuged at 11,790 x g for 1 hour. The virus banded 2 to 3 cm below the surface. The layer above this

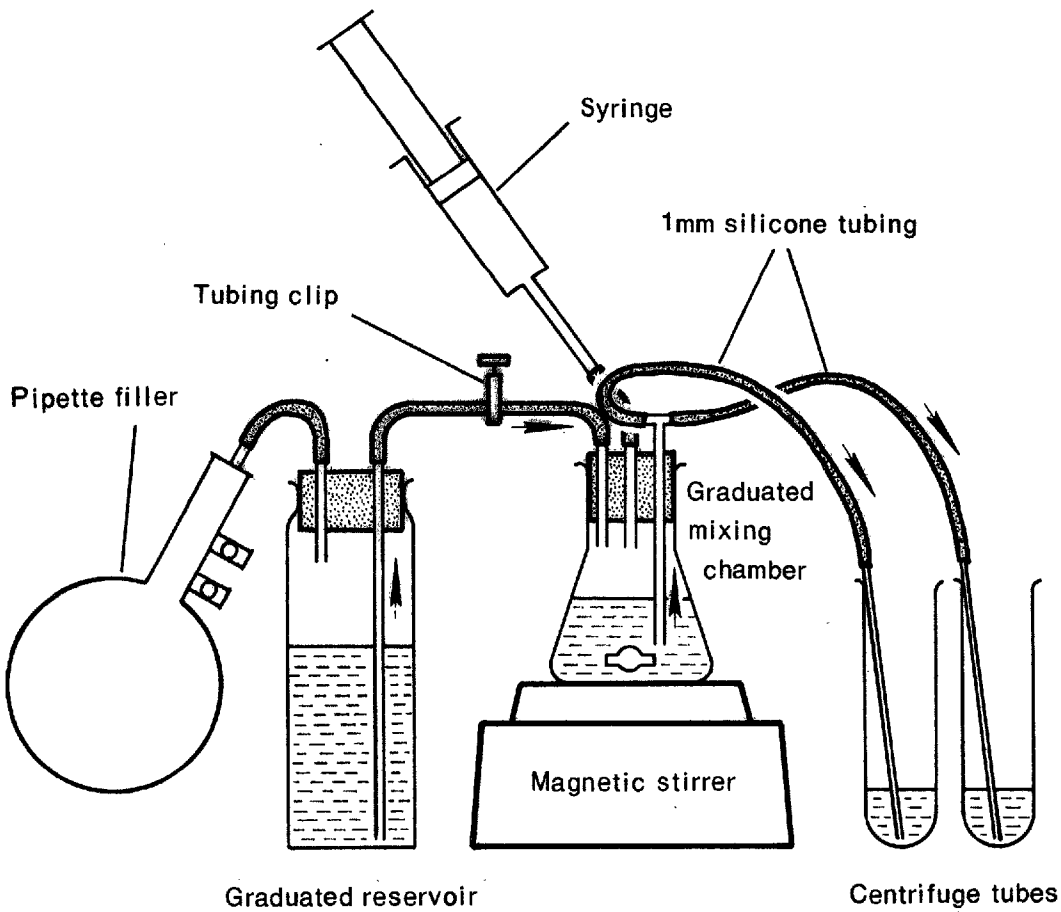


Fig. 1

Apparatus employed for the preparation of continuous sucrose density gradients. (Adapted from McCall and Potter, 1973).

band was removed using a suction tube consisting of a second centrifuge tube fitted with a rubber bung with 2 glass tubes, one of them bent and finishing in a fine point the other attached to a variable suction pump. After the upper layer had been slowly sucked into the second tube, the virus band was removed by a Pasteur pipette with a fine end bent at right angles. The virus was pelleted, resuspended in distilled water and further purified on a second sucrose gradient.

The virus so obtained was washed 3 times in distilled water using repeated cycles of centrifugation and maintained in a buffer solution used to avoid clumping of the virus (Martignoni et al., 1971). This consisted of sodium chloride 9 g/l, dioctyl sulfosuccinate sodium salt, 7.5 mg/l in tris buffer 0.01 M, pH 7.0.

(iii) Estimation of the Number of Capsules in the Suspension.

A Helber Counting chamber with a depth of 0.01 mm was used to count the number of virus capsules present. In this chamber the graduations divide an area of 1 mm^2 into 400 squares. The counting chamber and cover glass handled with a pair of fine forceps were rinsed in alcohol and wiped with lense tissue paper. About 1 µl of the sample was deposited in the chamber and the cover glass was carefully lowered into position and gently pressed down until Newton rings appeared where the cover glass rested on the surface of the slide around the chamber. Twenty squares were selected at random

and the capsules counted with a phase contrast microscope using a 10x eyepiece and 40x objective. The chamber was then refilled and the entire procedure repeated 4 times. Before counting the capsules it was necessary to dilute the suspension. Trials showed that by diluting the virus preparation in distilled water 1:1000 the number of particles reached a level at which counting was possible. An estimated 8.11 ± 2.22 standard deviation (S.D.), capsules per square were obtained providing 3.2×10^8 particles per microliter in the purified suspension.

3. A Colorimetric Method of Measuring Food Consumption.

The quantity of food consumed by individual larvae varies and an attemtp was made to devise a suitable colorimetric method by which food consumption could be measured.

The following procedure was adopted to test the compounds. A cabbage leaf disk 1.9 cm in diameter was cut with a number 13 cork borer. The stains and indicators were dissolved or suspended in distilled water containing 0.2% Teepol L to increase the wetting power of the suspension and 5 µl was applied to each disk which was then allowed to dry naturally.

Ten newly moulted second-instar larvae were placed in a 5 x 2.3 cm glass tube and the disk with the treated surface downwards was pinned to the filter paper seal and the tube maintained vertically. The perimeter of the leaf disk was left partially touching the tube wall so that the larvae

could easily reach the leaf. The faecal pellets were collected in the bottom of the tube and later suspended by adding 5 ml of distilled water except in the case of phenol red where 0.02% sodium hydroxide was used. In the case of phenol red,(the dye ultimately adopted), recovery was measured in a Pye Unicam SP 500 or a SP 30 Spectrophotometer.

4. Bioassays.

(i) Determination of the LD_{50}.

In order to ensure that a known dose of virus is ingested by each individual larva the leaf disk method was adopted (Morris 1962, McEwen and Hervey 1958). Disks of cabbage leaves 5 mm diameter were cut with a number 2 cork borer, for third-instar larvae and of 9 mm diameter with a number 4 cork borer for fourth-instar larvae.

Four dosages of virus were then applied to these disks, 6.4×10^5, 1.2×10^6, 6.4×10^6, 1.2×10^7 capsules per disk in a volume of 2 µl for third-instar and 1.6×10^6, 1.6×10^7, 1.6×10^8 and 1.6×10^9 capsules per disk in a volume of 5 µl for fourth-instar larvae. The dose was placed on each disk with an Agla micrometer syringe, and the droplet carefully spread with a 0.12 mm thick wire loop. Once dry a disk was given to a single larva confined in a 5 x 2.3 cm tube with a filter paper lid. Newly moulted third- or fourth-instar virus-free larvae reared on standard diet were starved for 2 hours before dosing. After 24 hours the larvae that

consumed the complete disk were transferred to untreated standard diet. Five larvae were confined in one tube and observations were stopped 5 days after the first larvae died of granulosis virus, for reasons that will be mentioned in the next section.

Mortality was assessed daily and dead and moribund larvae were removed.

(ii) Measurement of Comparative Susceptibility of Four Different Stocks of P. brassicae.

Two larval instars of each stock were used to compare the susceptibility to granulosis virus of the 4 stocks of P. brassicae. The larvae were reared on standard diet in one pound jam jars in groups of 200 in each, under similar conditions to the virus-free stock.

Larvae just moulted to the second-instar, of the virus-free, French, Dutch and cheiranthi stocks were transferred to a plastic box and starved for 2 hours and then transferred to diet treated with virus following the procedure described by David et al., (1971b). This consists of spreading 0.1 ml of the virus suspension per tube over a shallow layer of formalin-free diet 3 cm in diameter tubes. The drying process was assisted by gentle infra red heat. Once dry 20 second-instar larvae were allowed to eat for 24 hours on the treated surface and were then transferred to fresh standard diet. Dead or moribund larvae were removed daily and the test in any one tube was stopped

5 days after the first larvae had died of virus. Deaths due to secondary infection were thus avoided : as it takes approximately 6 days for a second-instar larvae to die with this dose and presumably another 6 days for those reinfected.

The two concentrations used in these tests were 3.2×10^5 and 3.2×10^3 capsules per tube.

For the estimation of the LD_{50} of fourth-instar larvae of the Dutch, French and cheiranthi stocks. The method described in section 4. (i) was followed. Four dosages were used, 1.6×10^8, 1.6×10^7, 3.2×10^6, 1.6×10^6 capsules per disk.

(iii) Effects of Different Treatments on Virus Viability.

The effect of the gut environment on the virus was studied by subjecting it to gut fluid, buffers and by bio-assaying it after it had been ingested by 3 species of Lepidopterous larvae.

For the gut fluid tests, gut fluid was obtained from fifth-instar virus-free larvae that had been fed cabbage during the last instar. The regurgitated gut fluid was collected in cooled 1.1 x 6.3 cm plastic specimen tubes previously frozen individually in a capped 2.7 by 7.6 cm polyethylene tube three-quarters filled with water. The larvae were made to vomit by lightly pressing and provoking them with sharp pointed tweezers. About 30 larva yielded 0.3 ml of gut fluid this varying considerably especially

with the duration of the preliminary starvation. Fifth-instar larvae, 24 to 72 hours old, regurgitated more readily than older larvae, and those starved for approximately 3 hours yielded more than those feeding or starved for longer periods.

The pHs of gut fluids were caused to vary by subjecting the larvae to various starvation periods or to no starvation at all. The pH of the gut fluid obtained was measured with small volume Probion electrodes described in section 5. (i).

To investigate the effect of the gut fluid on the virus, 50 µl were deposited in a 1.5 ml round bottom glass vial and 5 µl of virus suspension containing 1.6×10^9 capsules was added and stirred with a fine glass rod. The reaction was stopped after 1 minute by adding 1 ml of 0.1 M citric acid phosphate buffer (McIlvaine) pH 6.8. This gave a pH near netrality which it was assumed would stop most effects due to enzymatic activity and alkalinity.

Treatments using gut fluid boiled for 5 minutes to denature enzymes were also included. The pH measurements were made on this boiled gut juice, just before it was mixed with the virus.

In another series of experiments buffers were used to test the effect of alkaline pH on granulosis virus. A range of alkaline conditions similar to those occuring in the alimentary canal of P. brassicae were produced by

using 0.1 M glycine/NaOH buffer (Sorensen-Walbaum). Fifty µl of the buffer were mixed with 5 µl of granulosis virus containing 1.6×10^9 capsules. After 1 minute the reaction was stopped by adding 1 ml of 0.1 M citric adid (McIlvaine) pH 6.8. Both buffers were prepared following the directions given by Grimstone and Skaer (1972).

The remaining virus activity of the suspension was bioassayed by spreading 0.1 ml on a formalin-free diet (David et al., 1971b). Ten tubes were set up per treatment with 20 newly moulted, second-instar larvae per tube. The mortality was recorded daily until the 10th day after dosing.

The passage of the granulosis virus through the alimentary canal and its persistence was investigated using larvae of P. brassicae and also Spodoptera littoralis (Boisduval)(Lep. Noctuidae) and Lacanobia oleracea (Linnaeus) (Lep. Noctuidae). All species were in their final instar. The larvae of P. brassicae were fed on standard diet and the latter two species on cabbage, prior to the administration of granulosis virus.

The virus used in this test was semi-purified as follows : Twenty milliliters of P. brassicae larvae which had died of granulosis, were triturated in a tissue grinder and strained through fine nylon mesh. The suspension was diluted in distilled water and centrifuged at 482 x g for 20 minutes. The supernatant was centrifuged at 13298 x g for 20 minutes and the pellet containing the virus was washed with distilled

water and recentrifuged twice. Finally the virus was suspended in 5 ml of 0.2% Teepol L in distilled water.

Cabbage leaf strips were cut to 1.8 x 7 cm and approximately 33 µl of the virus suspension were applied to each strip and distributed with a fine camel hair brush. One larvae was placed in a 7.5 x 2.5 cm glass tube, sealed with filter paper, and allowed to feed for 18 hours. The gut contents of each larvae were then separately bioassayed. The P. brassicae larvae were anaesthetized with a drop of ether in a petri dish and then immersed in a 5% sodium hypochlorite solution for 1 minute to inactivate the virus that might be adhering externally to the larvae and rinsed with distilled water. The complete alimentary canal was then dissected and cut into 5 sections. In between each cut the scissors were rinsed first in 5% hypochlorite and then in distilled water. The S. littoralis and L. oleracea larvae were frozen in liquid nitrogen and the contents of the alimentary canal sections dissected out.

For the 3 species the following sections were cut :-

1) Crop
2) anterior part of mid-gut
3) medium part of mid-gut
4) posterior part of mid-gut
5) ileum and rectum

Each section was ground in a watch glass in 0.1 ml of citric acid/phosphate buffer pH 4.7 to bring the pH near

neutrality and stop further inactivation ; 0.3 ml of distilled water were added and the resulting suspension was distributed with a brush on the surface of 3 formalin-free diet tubes 7.5 x 3.0 cm. Twenty newly moulted second-instar larvae per tube were allowed to feed for 24 hours on treated diet and then transferred to standard diet. Mortality was recorded for the following 10 days.

Parallel to this bioassay, the pHs of the alimentary canals of 2 S. littoralis and 3 L. oleracea larvae fed on untreated cabbage were recorded. The alimentary canal was divided into the same 5 regions and the pH measured as described in section 5. (i).

(iv) Action of Diet Additives on Mortality of Virus Infected Larvae.

If the gut environment in which the virus invades the columnar cells is made more suitable for infection it can be assumed that an increase in granulosis virus mortality should become apparent.

(a) Acid Diet Tests.

In this test the pH of the diet was lowered by applying chemicals to its surface. Tubes measuring 7.5 x 3.0 cm with a shallow layer of formalin-free diet were treated with 0.1 ml of aqueous solution of the chemicals as shown in Table 1. Three hours after this treatment the pH of the diet surface was measured by removing a layer 1 mm deep and depositing it between two low volume Probion Electrodes

Mg per tube				
Citric acid	plus	Disodium hydrogen phosphate	pH of the solution prepared	pH of the diet surface
8.3		3.6	3.1	5.2
16.67		7.3	3.05	4.85
83.3		36.6	3.0	4.1
102.9		3.91	1.8	3.9
Sodium hydrogen sulphate				
5			0.9	5.6
15			0.6	4.95
Oxalic acid				
1			1.43	5.95
2			1.25	5.8
5			1.0	5.2
6			0.9	5.2
8			0.8	4.9
12			0.7	4.75
Control			---	6.2

Table 1

Amount of chemicals added per tube and pHs of the solutions and of the surface of the diet.

(Table 1).

Soon after the diet surface had dried 0.1 ml of granulosis virus suspension, containing 3.2×10^5 capsules was applied evenly to each of 10 tubes and when this had also dried, 20 newly moulted second-instar larvae were allowed to feed on the diet for 24 hours and then transferred to untreated standard diet. A set of 10 control tubes with diet treated with virus only was included for each batch of larvae. Mortality was recorded until the 10th day after infection.

In the case of oxalic acid its direct effect on the virus was also tested. Virus suspensions containing 3.2×10^3 capsules per µl were prepared containing 0.1, 1, 5 and 10% w/v, of oxalic acid and maintained in the dark, at room temperature, for 24 hours. The solutions were bioassayed as previously described on formalin-free diet. After 24 hours on this surface the larvae were transferred to new standard diet. Mortality was assessed until the 10th day after dosing.

(b) Trypsin Inactivators.

Tests were made to determine whether inactivation of the granulosis virus in the alimentary canal was also due to proteolytic enzymes in the gut fluid. To test this assumption an attempt was made to inhibit these enzymes. Lecadet and Dedonder (1966) have shown that trypsin and

chymotrypsin of P. brassicae are partially inactivated by a trypsin inhibitor extracted from soybean. A second inactivator, Trasylol which is a general inhibitor of trypsin, chymotrypsin and other enzymes (Auhagen 1966 and Werle 1972) was also tested.

Soybean Trypsin Inactivator, salt free, lyophilised was used to prepare 0.01, 0.1 and 1% dilutions, and Trasylol 0.05, 0.1, 1 and 10% w/w dilutions both in distilled water and 0.1 ml of these solutions were spread over a shallow formalin-free diet per tube. A control of the 10% solution of Trasylol was included to observe the toxicity in larvae (180 used). Once absorbed in the diet 0.1 ml of virus suspension containing 3.2×10^5 granules was dispersed over the treated diet. Ten tubes were used per concentration of each inactivator. A treatment dosed only with virus was included as a comparison with each batch of insects. Twenty newly moulted second-instar larvae were allowed to feed for 24 hours and removed to normal diet. Mortality was recorded until the 10th day after dosing.

5. Histological and Physiological Measurements of the Larvae.

(i) pH Measurement in the Alimentary Canal.

The importance of pH to the stability of virus in different environments is reviewed by Yendol and Hamlen (1973) and more specifically in the gut by Tinsley (1975).

A method of measuring the alimentary canal pH of P. brassicae larvae was devised. They were reared as usual on standard diet but changed to cabbage from the beginning of the instar in which the measurements were made.

Fourth instar larvae which had been feeding on cabbage for 24, 48, 72 hours were placed in aluminium mesh rolled into a tube and quickly submerged in liquid nitrogen. The tube prevented the larvae from curling and maintained them in nearly their normal posture. Once completely frozen the larvae were taken out of the mesh and stored in a metal dish placed in the mouth of a vacuum flask containing liquid nitrogen. This treatment rapidly killed the larvae and prevented further reactions in the gut that could alter the pH. It also enabled the larvae to be cut in sections so as to obtain more details of pH changes along the length of the mid-gut. At a particular temperature, still below $0^{\circ}C$, the section may be cut and the surrounding integument and gut wall easily peeled away, leaving only the gut contents.

The sections were cut under a binocular microscope. The larvae were placed in a plastic petri dish cooled by placing under it a small insulated dish filled with a sponge saturated with liquid nitrogen. Every so often the sponge was resaturated.

The larvae were cut between each segment as indicated in Fig. 2.

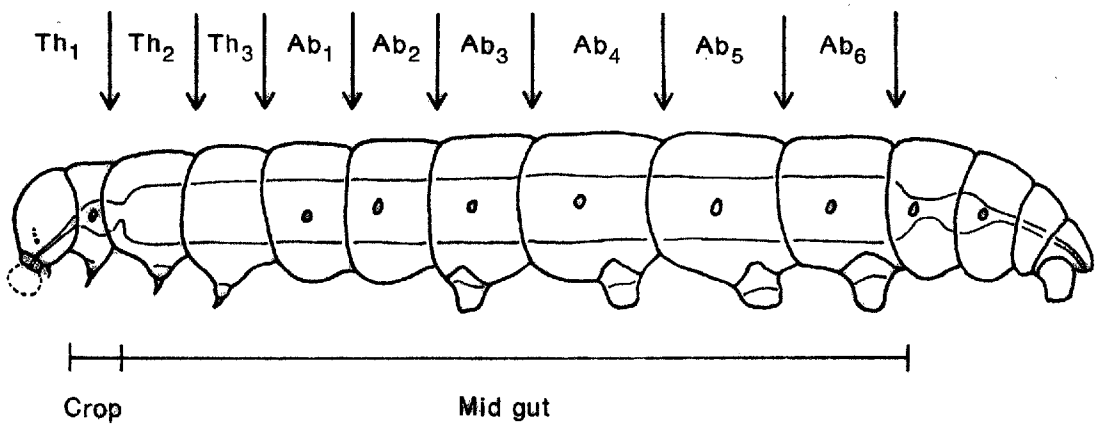


Fig. 2

Schematic diagram of a larva indicating segmentation and where cuts were made (indicated by arrows) when measuring the pH of the crop and midgut of *P. brassicae* larvae.

Measurement No.	Section of the alimentary canal	Anatomic segment involved
1	Regurgitated fluid	Mouth parts
2	Crop	First thoracic segment
3	1st section of mid-gut	Second thoracic segment
4	2nd section of mid-gut	Third thoracic segment
5	3rd section of mid-gut	First abdominal segment
6	4th section of mid-gut	Second abdominal segment
7	5th section of mid-gut	Third abdominal segment
8	6th section of mid-gut	Fourth abdominal segment
9	7th section of mid-gut	Fifth abdominal segment
10	8th section of mid-gut	Sixth abdominal segment

Table 2

pH measurements of the gut contents. Division into sections of the alimentary canal of P. brassicae related to the external anatomy of the larvae.

When a larva was plunged into the liquid nitrogen it regurgitated a variable amount and the pH of this frozen gut fluid was also measured.

In each larva 10 pH recordings were made as described in Table 2. For each of these a piece of gut contents was placed between the two electrodes connected to a Pye model 79 pH meter and as soon as it thawed the pH was recorded. The small volume electrodes (around 0.05 ml total volume) were manufactured by Probion Limited.

The same procedure was used to measure the pH of fifth-instar larvae, except that the dissection was carried out on a small petri dish filled with wax, under a binocular microscope. Cooling during dissection was not necesary because the fifth-instar larvae thawed more slowly and could be dissected without difficulties. Measurements of the pH were taken 24, 48, 72, 96 and 120 hours after moulting when testing the virus-free fifth-instar larvae and after 72 hours with the Dutch, French and cheiranthi stocks.

(ii) Histological Observations on the Peritrophic Membrane

The importance of the peritrophic membrane as a barrier to pathogens that would invade the gut cells is generally agreed, (eg. Orihel 1975) although it has not been shown in the case of virus to which extent this membrane acts as a barrier. Tinsley (1975) briefly mentions its importance as does David (1977).

This study was planned to obtain information about the formation and origin of the peritrophic membrane, by using light microscopy.

Healthy larvae of the virus-free stock were fed on cabbage leaf. Leaf was chosen because it maintains better the original volume of the gut, is the natural food and contrasts well with the insect tissues when viewed in light or electron microscopes.

The alimentary canal was dissected from anaesthetised larvae under a binocular microscope and fixed and dehydrated following a procedure described by Martignoni (Personal communication). The complete alimentary canal was placed overnight in a mixture of 12 ml of 25% glutaraldehyde and 8.8 ml Sorensen's phosphate buffer pH 6.6. Afterwards it was rinsed in Sorensen's phosphate buffer pH 6.6 and post-fixed for 2 hours in a solution of

Osmium tetroxide (Os_5O_4) 2% - 5 ml
Sorensen's phosphate buffer pH 6.6 - 5 ml

The alimentary canal was then rinsed in the same buffer and dehydrated in ethyl alcohol as follows :

25% ethyl alcohol 10 min
50% ethyl alcohol 10 min
75% ethyl alcohol 10 min
100% ethyl alcohol 15 min repeated twice.

For light microscopy the gut was placed in n-butyl methacrylate directly from the last ethyl alcohol and changed three times to fresh n-butyl methacrylate containing 1% w/v of the hardener benzoyl peroxide, and finally embedded in a mixture containing 1.5% w/v of the hardener, in polyethylene capsules. Polymerization was achieved by uv light, by keeping the capsules 4 cm from a common fluorescent tube for 3 days (Pease 1964).

Sections 1 micron thick for the light microscope were obtained in a LKB Piramitome with glass knives. The sections were floated on a drop of water on the slide and heated to 60°C for 2 hours on a dish warmer. This stretched and anchored the sections to the slide. Staining was achieved with 1% methylene blue in 1% borax mixed, in equal amounts just before use, with 1% aqueous azure II or alternatively with 1% toluidine blue in water 1 ml, plus 20 ml 25% sodium bicarbonate passed through a GS 0.22 μ Millipore filter before use (Dawes 1971).

(iii) Measurement of the Diameter of the Mid-Gut.

The principle that the smaller the body, the bigger the surface per unit of volume enclosed is well documented (Crowe and Crowe 1969) and was analysed in relation to the diameter of the mid-gut in different larval instars of the virus-free stock of P. brassicae. First-instar larvae just hatching from the egg and 2 to 3 hours older were dissected under a binocular microscope and the peritrophic membrane

carefully pulled out, and mounted in water on a slide with a cover glass. The peritrophic membrane thus flattened was measured under a microscope and its width was assumed to be half the circumference from which the diameter was calculated. Altogether 18 first-instar larvae were measured.

The alimentary canals dissected from the larger larvae were placed in Bouin's fixative overnight (Grimstone and Skaer 1972). They were then dehydrated by passing through alcohol of increasing concentration and cleared in carbon tetrachloride. Finally the complete alimentary canal was embedded in Paraplast at 60°C with three changes of wax.

Sections of the median region of the mid-gut were cut with a Cambridge Rocking Microtome 6 to 10 microns thick. The ribbons were mounted on slides and stained in Carazzi's haematoxylin (Disbrey and Rack, 1970) or Mann's methyl blue/ eosin (Grimstone and Skaer 1972).

The diameter of the peritrophic membrane was measured as well as the inner diameter of the gut wall, measurements were made on 15 larvae from each instar.

(iv) Measurement of Faeces Production.

If the food passes through the alimentary canal more quickly it could be argued that the virus has a shorter time to infect the columnar cells which could result in a change of susceptibility to virus. Previous researchers have

shown that there is a high correlation between the amount of food ingested and the number of faeces produced (Ito 1960). As a measure of the rate which the food passes through the gut the excretion rate was recorded.

RESULTS

1. The Colorimetric Method for Determining Food Consumption.

(1) Preliminary Tests with Various Dyes and pH Indicators.

A useful compound should have the following properties :

1) not be absorbed or decomposed by the insect gut
2) be readily extracted from the frass
3) not be bound to the vegetable surface or food
4) not be toxic to the insect or pathogens
5) without effect on the quantity of food consumed
6) strongly colour the water extractions

Most of the stains failed in one or more points and were not further tested. The observed properties of the tested materials are given in Table 3.

Nigrosin G 140, nigrosin nichrome, carmine, edicol supra black, toluidine blue and sudan III were not recovered from the frass. The remaining compounds were recovered up to a variable extent. Neutral red and nile blue stained the entire larvae red and blue respectively. Carmine and alcoholic eosin crystals stained the malphigian tubules slightly pink. Orange G formed crystals as it dried on the leaf surface, which redissolved with difficulty. Phenol red was easily recovered from the frass by employing a 0.02% Na OH solution and dissection of the larvae revealed that it did not stain the gut wall or other tissues.

TABLE 3

Compounds fed to P. brassicae larvae, recovery in the frass and staining of the gut wall.

Stains or pH indicators	Colour Index Number 1956/1971 or pH range	Concentration % (w/v)	Effect on gut wall	Recovery from frass
Azocarmine (Gurr)	50085	2.5	not stained	Partially
Carmine (BDH)	75470	5	pale orange	Not recovered, pellets stained
Congo red (Gurr)	22120	1	not stained	Partially
Edicol supra black (ICI)	-	5	not stained	Not recovered
Edicol supra tartrazine (ICI)	-	5	not stained	Partially
Edicol supra yellow (ICI)	-	5	orange→yellow	Partially
Eosin alcoholic (BDH)	45386	1	pale pink	Recovered in alcohol
Light green (Gurr)	42095	1	not stained	Partially
Lissamine Fast Yellow (ICI)	-	5	not stained	Partially
Neutral red (BDH)	50040	1	red	Partially
Nigrossin G.140 (ICI)	-	10	not stained	Not recovered
Nigrosin Nichrome (Gurr)	50420	5	not stained	Not recovered, Gut contents black

Continued on next page

TABLE 3 (continued)

Compounds fed to P. brassicae larvae, recovery in the frass and staining of the gut wall.

Stains or pH indicators	Colour Index Number 1956/1971 or pH range	Concentration % (w/v)	Effect on gut wall	Recovery from frass
Nile blue (BDH)	51180	1	blue	Partially
Orange G. (Gurr)	16230	5	not stained	Partially
Sudan III (BDH)	26100	1	not stained	Not recovered
Tartrazine (ICI)	-	5	yellow	Recovered
Toluidine Blue	52040	1	pale blue	Not recovered
Bromo cresol purple	pH 5.2-6.8	1	not stained	Partially
Phenol red water soluble	pH 6.8-8.4	1	not stained	Recovered

(ii) The Phenol Red Method.

(a) Optical Density.

Optimal spectrophotometric absorbance was determined for five concentrations of phenrol red from 0.0001 to 0.0009% in 0.02% sodium hydroxide which dissolved the phenol red more readily than water and gave an intense red colour to the solution. The optical density for each concentration was recorded at 15 different wavelengths using a Pye Unicam SP 500 Spectrophotometer with 5 ml glass cells and a tungsten filament light source. The curves obtained are shown in Fig. 3. All 5 solutions showed similar absorption curves, each with a maximum at 560 nm.

The optical density curve was established at this wavelength by preparing 15 solutions ranging from 0.001 to 0.00001%. Fresh dilutions were made and the determination was replicated 3 times. Each point of the curve of Fig. 4 was based on the mean of these replicates. A simple regression analysis gave a correlation coefficient r = 0.9999 and the equation for the straight line y = 0.00059x

The conversion to the amount of diet containing phenol red consumed was calculated by the following formula :

$$\frac{\text{ml used in suspending the frass} \times \text{Optical density of the solution (frass)} \times 0.00059}{\text{\% of phenol red in the diet}} = \text{gr of diet consumed}$$

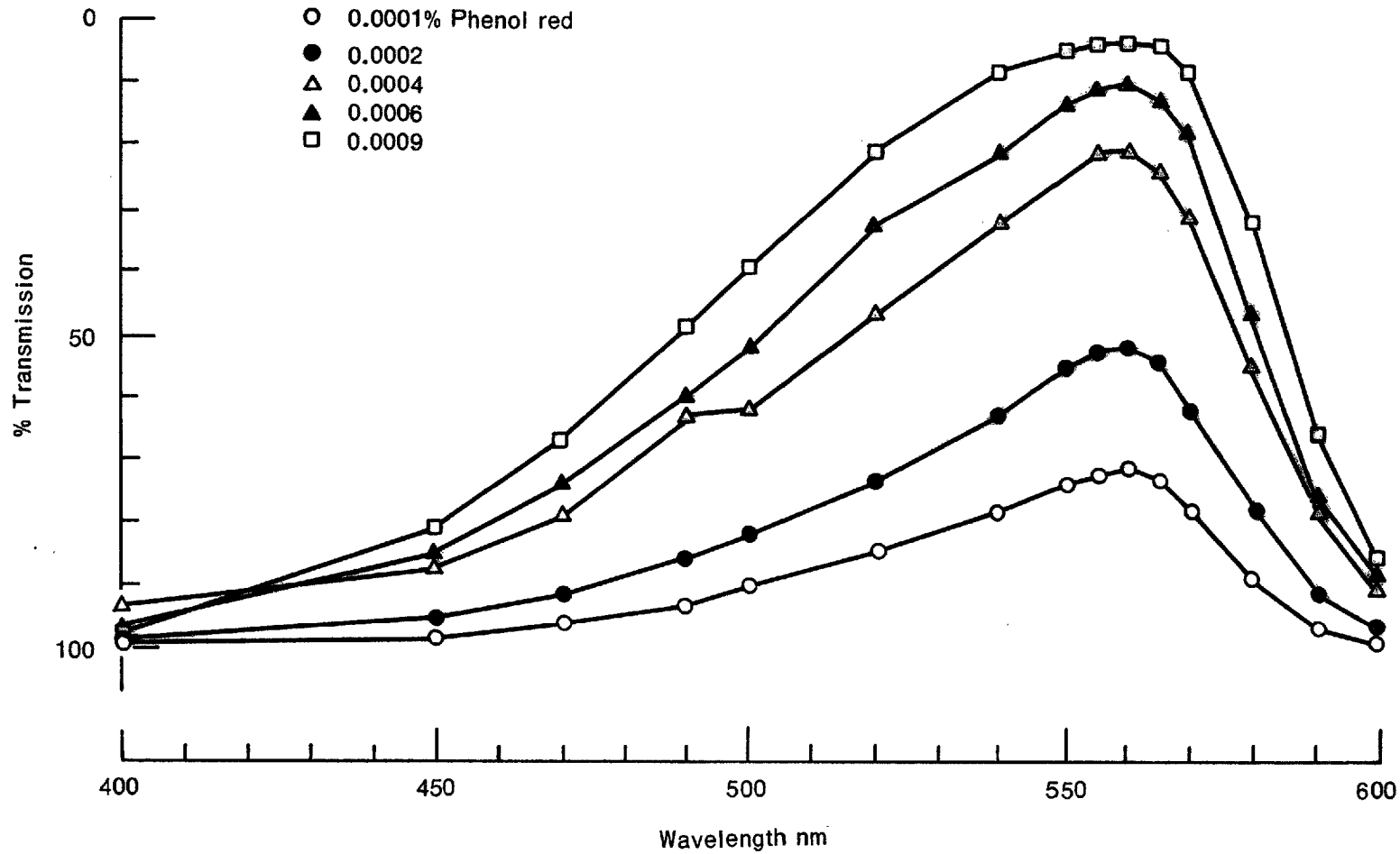
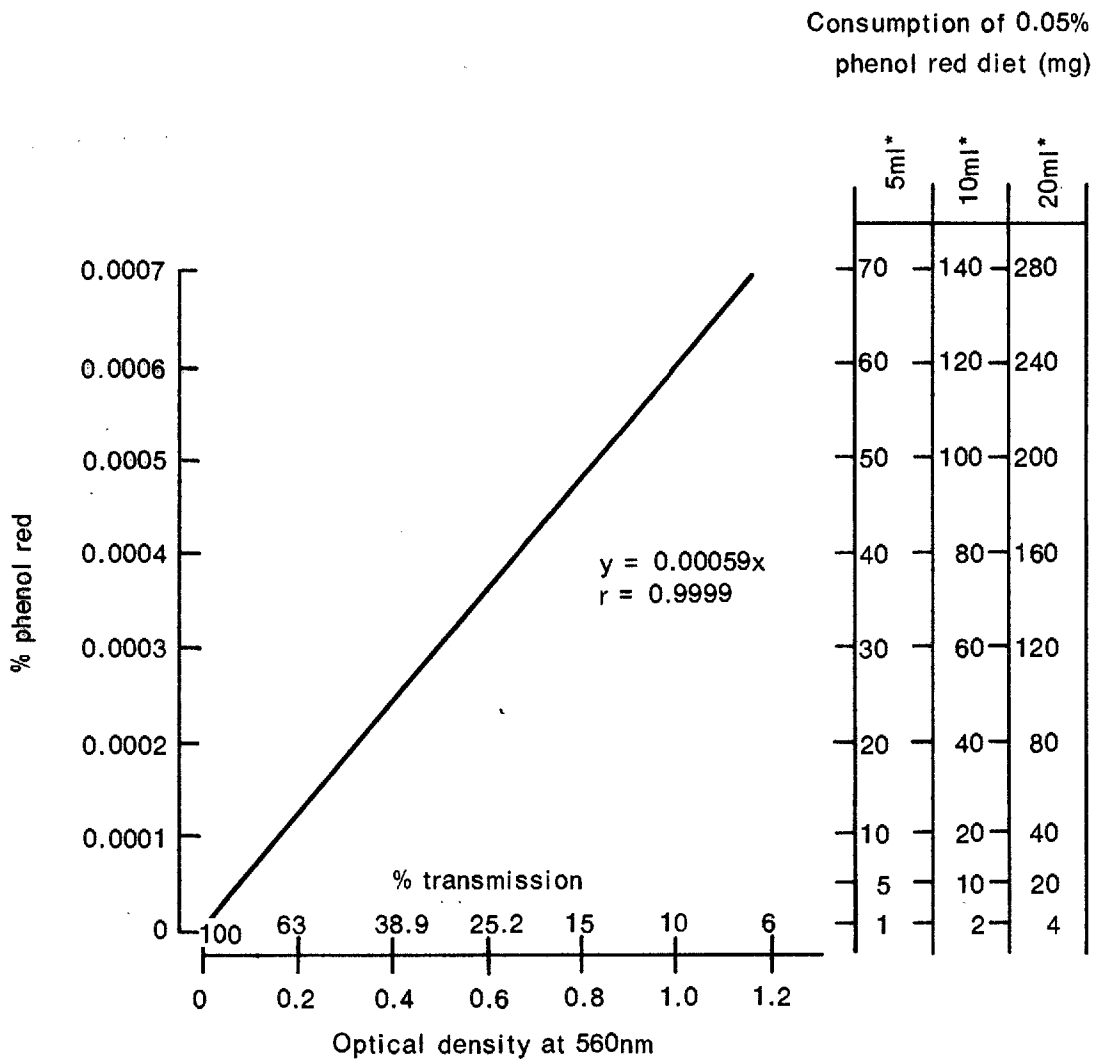


Fig. 3

Optimal wavelength absorption of phenol red in 0.02% sodium hydroxide.



* Volume used to suspend the frass

Fig. 4

Optical density of phenol red solutions and direct transformation to diet consumption.

As the volume of sodium hydroxide used in suspending the frass and the % of phenol red in the diet becomes standard in a test, ultimately the above formula may be simplified to a constant which is multiplied by the density of the frass suspension which then gives directly the amount of diet consumed.

(b) Recovery from Leaf and Frass.

The retention of phenol red by the leaf tissue and the amount lost by absorption in the alimentary canal was investigated. Four different treatments were undertaken.

a) a disk of cabbage leaf 1.9 cm in diameter was painted with 5 µl of a 0.5% phenol red in 0.1% Teepol L solution. Once dried it was suspended inside a tube, pinned to the filter paper lid as previously described and 20 newly moulted second-instar larvae were allowed to feed for 24 hours on the disk. They were then transferred to a fresh unstained disk of cabbage for 48 hours to evacuate all the phenol red remaining in the alimentary canal.

b) only 10 larvae were allocated per tube, following the same procedure,

c) treated leaf disks were allowed to dry for 24 hours and the phenol red was redissolved in 0.02% sodium hydroxide to determine the recovery from the leaf.

In each case the frass, the remaining piece of disk, or the complete disk was added to 10 ml of 0.02% Na OH

in distilled water to extract the phenol red. For the 3 foregoing tests 5 µl of the 0.5% phenol red solution diluted in 10 ml was used as a control. The light absorption was measured at 560 nm as before.

Results showing the recovery of phenol red in the 4 treatments are presented in Table 4. The statistical analysis was carried out on the original (crude) transmission data. This was done to avoid a larger error involved in converting each measurement into amount consumed.

The amount of leaf consumed varied more with 20 than with 10 larvae per disk as shown by the S.D. of the transmission of the frass extracts.

Once phenol red dried on the cabbage disk, 95% was recovered compared to the control by washing it off with the sodium hydroxide solution and 95 and 91% was recovered from the 10 and 20 larvae treatments respectively.

(c) Toxicity Assay.

In order to determinate whether phenol red had any noticeable toxic action, larvae were reared from first- to fifth-instar on standard diet containing either 0.01% or 0.05% phenol red. To prepare these diets the phenol red was first dissolved in part of the water used for making up the standard diet. Larvae were set up in groups of 20 per tube replicated 10 times in each treatment. Control larvae were maintained on standard diet without phenol red. The

TABLE 4

Recovery of phenol red from larvae fed on cabbage disks treated with the stain.

Treatment	No. of replicates	Transmission (%)				Phenol red recovered (mg)	
			Mean	S.D.	95% Confidence interval for the mean	Partial	Total
20 larvae per disk	5	Frass	77.8	3.08	74.01-81.67	0.0065	0.019
		Uneaten piece of disk	60.48	2.73	57.09-63.87	0.0129	
10 larvae per disk	5	Frass	84.77	0.59	84.04-85.50	0.0044	0.020
		Uneaten piece of disk	53.96	2.21	51.22-56.70	0.0157	
Dried disk	10	-	45.45	1.42	44.44-46.46	-	0.020
Phenol red calibration control	6	-	43.6	0.85	42.75-44.55	-	0.021

following observations were carried out in this test ; adult emergence from 200 first-instar larvae ; the weight of 50 randomly chosen pupae ; egg batches laid per approximately 200 adults ; general fertility of the eggs.

Results from Table 5, suggests that feeding of phenol red at the 2 concentrations tested did not affect mortality, the weight of two day old pupae, oviposition or fertility. (The latter was visually examined and appeared equal to the controls).

First- and second-instar larvae were slightly reddish due to the red contents of the alimentary canal. Older larvae showed a darker colour compared to controls due to their less transparent cuticle. No abnormal pigmentation, deformation or wing defects were observed among the pupae or adults.

Concentration of phenol red in the diet	Initial No. of 1st instar larvae	Mean weight of 50 pupae 2 days old (mg)	Adults emerged	No. of egg batches laid in 3 days
0.05	200	373.0	198	77
0.01	200	368.7	194	72
Control	200	370.1	194	71

Table 5

Effect of feeding the larvae on diet containing phenol red on mortality, pupal weight and oviposition.

In the above Table the 2 variates, adults emerged and mean weight of pupae were statistically analysed. Simple chi square (χ^2) test showed that there was no heterogeneity between the tubes within each of the 3 treatments. A second χ^2 test revealed there were no apparent differences between the total number of adults emerging from each treatment. For the mean weight of pupae simple one way analysis of variance revealed no significant difference between treatments.

(d) Compatibility with Granulosis Virus.

The storage stability of virus in the presence of phenol red was tested. Two virus suspensions were used, 3.2×10^3 and 3.2×10^4 capsules per µl, suspended in buffer solution (Martignoni et al., 1971) and phenol red was added to make up a 1% w/v solution. These preparations and a control suspension without phenol red were maintained in darkness at 20 ± 1°C for 9 days. All the suspensions were then bioassayed with second-instar virus-free larvae following the procedure described by David et al., (1971b) and outlined in section 4 (ii) of Materials and Methods. Ten tubes were set up for each suspension giving a total of 200 larvae.

Results (Table 6) show that under the conditions described phenol red did not reduce the viability of the virus. In fact a higher mortality was observed with the virus which had been stored with phenol red compared to the

control of the same virus concentration.

Phenol red concentration (%)	Dosage capsules/tube	No. of larvae treated	Mortality (%)	
			Virus deaths	Other causes
1	3.2×10^6	200	92.5	0
1	3.2×10^5	200	31.5	2.5
Control	3.2×10^5	200	19.5	5

Table 6

Effect of phenol red on the activity of granulosis virus after 9 days storage in 1% phenol red (As bioassayed with second-instar P. brassicae larvae.)

All three treatments were significantly different, χ^2 analysis at P = 0.01 .

(e) Sensitivity of the Method.

To determine whether small changes in the content of phenol red in the diet could be detected with this method standard diet was mixed with varying concentrations of the dye. Each time 50g of diet in a tube was melted in a hot water bath and 3 ml phenol red solution in distilled water was mixed in which a spiral wire plunger. The following phenol red concentrations in diet were prepared : 0.04, 0.05, 0.06, and 0.07% and four tubes per concentrations were each filled with 10g of diet. Twenty newly moulted second-instar larvae were then allowed to feed for 24 hours

in each tube. During this time the tubes were inverted, with the diet uppermost, and the frass fell on to a piece of aluminium foil that served as a lid. A small sachet made of 0.6g of activated silica gel wrapped in aluminium foil was left inside each tube to absorb excess moisture and prevent the faecal pellets sticking to the glass walls.

The pellets from each tube were then brushed off the aluminium foil into a 20 ml glass tube and suspended in 10 ml of 0.02% sodium hydroxide in distilled water. The absorption was measured at 560 nm in a PYE Unicam SP 500 Specrtophotometer. No corrections were made for the frass interference for reasons considered in the discussion.

The test showed a high variability between replicates (Table 7), especially at the 0.05% phenol red concentration as shown by the S.D.

The average larval consumption at all 4 concentration of phenol red diet varied from 30.6 to 39.3 mg with a considerable scattering of the S.D. Further statistical analysis and determination of the sensitivity of the method was therefore not possible. There were however indications of an increasing percentage of phenol red in the solutions as the concentration of the dye in the diet rose, except in the 0.07% concentration.

(f) Food Consumption Tests.

(A) During the first 24 hours of each Instar.

For these tests standard diet containing

TABLE 7

Recovery of phenol red from faeces of 20 second-instar P. brassicae larvae which had fed on diet containing 4 different concentrations of the dye.

Phenol red concentration (%)	No. of replicates	Transmission (%)			Phenol red in frass suspension (%)	Weight of diet consumed by larvae (mg)
		Mean	S.D.	95% Confidence interval of the mean		
0.04	4	56.8	2.28	53.17-60.42	.000122	30.6
0.05	4	53.2	7.04	42.0 -64.39	.000159	31.8
0.06	4	39.85	1.06	38.16-41.53	.000236	39.3
0.07	4	41.35	2.07	38.04-44.65	.000227	32.4

0.05% of phenol red was used. A drop of about 1g of the melted diet was deposited on one side of the bottom of a 5.0 x 2.3 cm glass tube and maintained slightly tilted until solidified to obtain the distribution of diet shown in Fig. 5. This position of the diet was adopted to reduce the surface exposed to dehydration and larval feeding. It also helped to prevent faecal pellets landing on the diet from which they could pick up phenol red.

The amount consumed in 24 hours by newly moulted second-, third-, and fourth-instar larvae was measured. Altogether 20 larvae, one per tube of each instar were set up. The frass produced was collected as described in the preceeding section and extracted in 0.02% Na OH. The results of these tests are summarised in Table 8. There was no overlap in the amount consumed in the different instars, indicating that the requirements in the first 24 hours are significantly different in the 3 instars measured. The mean amount consumed by a fourth-instar larvae was nearly 10 times as much as by a second-instar larva and 4 times as much as by a third-instar larva.

(B) <u>During each Complete Instar.</u>

The same method was adopted as in section (A) except that the amount consumed during each instar was measured using 20 second-,15 third-, 20 fourth- and 20 fifth-instar larvae. The silica gel sachets were replaced daily by reactivated ones. In this test the larvae were kept in

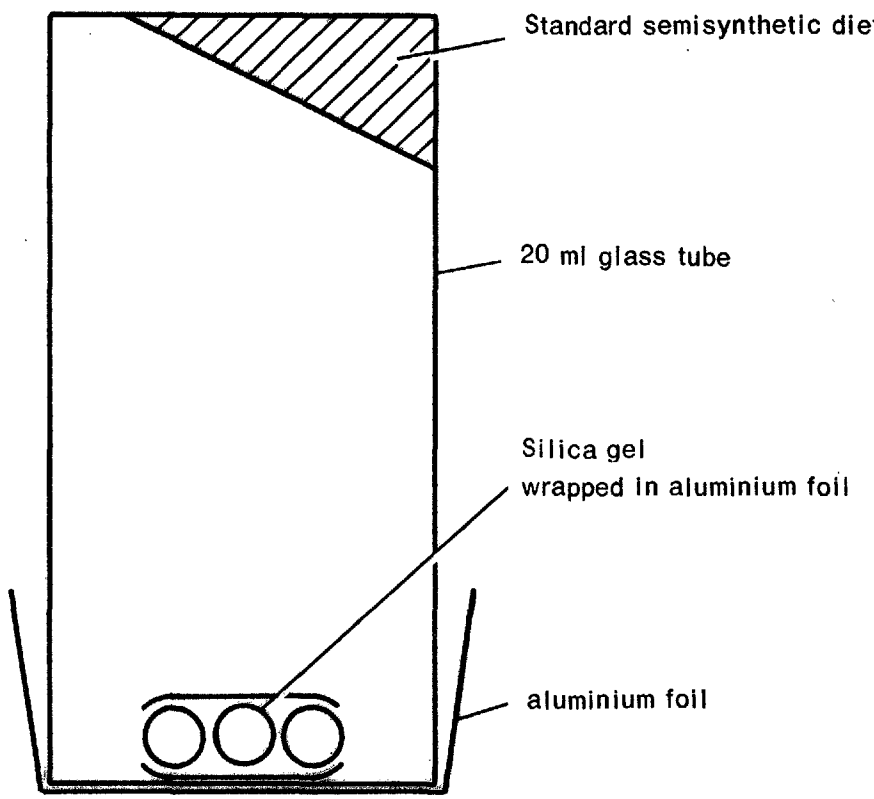


Fig. 5
Set up of the diet containing phenol red.

TABLE 8

Diet consumption of 20 larvae during the first 24 hours by second-, third- and fourth-instar P. brassicae larvae fed on 0.05% phenol red diet at 20 ± 1°C.

Larval instar	Volume of NaOH used to extract frass (ml)	Transmission (%)			Diet consumed (mg)	
		Mean	S.D.	95% Confidence interval for the mean	Mean	Range
2nd	5	84.17	4.29	82.16-86.16	4.5	2.3 - 7.0
3rd	5	66.55	4.68	64.36-68.74	10.43	7.0 - 13.5
4th	10	42.25	6.38	39.6 -45.52	43.8	30.6 - 61.3

the tubes until they were ready to moult and fifth-instar larvae were kept until pupation and weighed when 2 days old to observe the correlation between the amount eaten and the weight of the pupae. First-instar larvae were also tested but the small amount of frass produced was difficult to handle and the phenol red colour could not be detected in a 5 ml cuvetter in the spectrophotometer.

The frass was suspended in larger volumes of liquid with each higher instar. In the fifth-instar 550 ml had to be used to dilute the phenol red in the frass to a suitable concentration. The average amount of diet consumed by a larvae from second-instar to pupation was 1486.9mg, 85.9% of it during the fifth-instar, which was also the most variable of the 4 instars considering food consumption as suggested by the S.D. and range in Table 9.

The correlation coefficient between weight of pupae and percent transmission for individual fifth-instar larvae was r = 0.869 which was significant at 0.1% (t test with 18 degrees of freedom (d.f.)). This relationship is plotted in Fig. 6. No differences between males and females were apparent, except for the mean weight being slightly higher for females.

(g) Consumption of Diet with and without Sugar.

David et al., (1972a) reported that larvae fed on sugar deficient diet before and during a 3 hour period of virus ingestion showed a significantly higher mortality due

TABLE 9

Diet consumption during second-, third-, fourth- and fifth-instar by larvae of *P. brassicae* measured by the phenol red method at 20 ± 1°C.

Larvae		Volume of Na OH used to extract frass	Transmission (%)			Diet consumed (mg)	
Instar	No. fed		Mean	S.D.	95% confidence interval of the mean	Mean	Range
2nd	20	5	69.91	4.22	67.94-71.88	9.1	6.6- 12.2
3rd	15	10	53.72	4.04	51.48-55.96	31.8	25.6- 37.7
4th	20	20	19.66	3.20	18.16-21.16	167.5	149.8- 189.9
5th	20	550	63.4	6.18	60.51-66.29	1278.5	551.6-1817.2

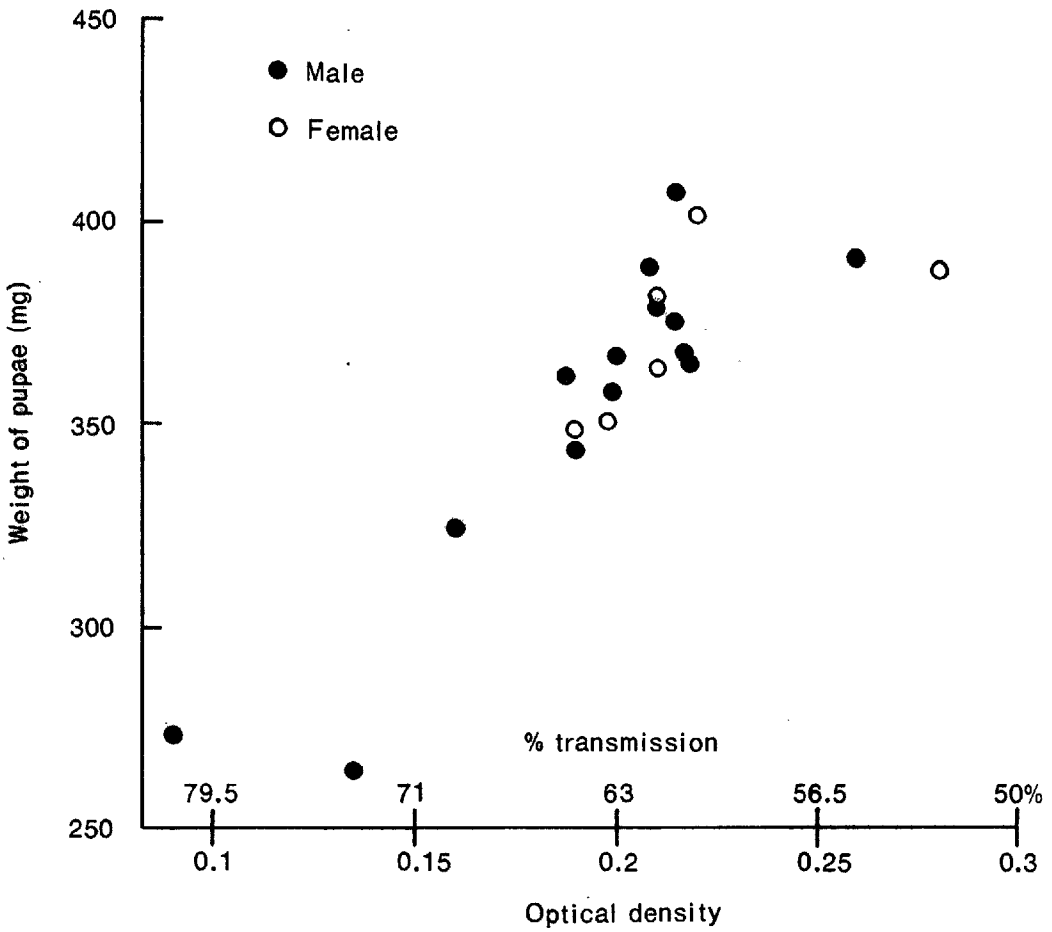


Fig. 6

The relatioship between phenol red recovery from frass of 5th instar larvae expressed in optical density and weight of the pupae.

to granulosis virus than larvae similarly treated on standard diet. It was therefore of interest to determine whether sugar had some effect on the food consumption of the larvae.

Standard diet to which 0.05% phenol red had been added was prepared with 3 concentrations of added sucrose.

a) Sugar deficient diet - no sucrose added

b) Standard diet - with 3.3% w/w sucrose

c) Sugar enriched diet - with 6.6% w/w sucrose

The sugar was dissolved in 1 ml of water and 50g of diet liquified in a hot water bath. It was thoroughly mixed with a spiral wire as used with the phenol red. About 2g of diet was then poured into each of 10, 7.5 x 2.5 cm glass tubes.

The effect of the sugar content on diet consumption was tested using second-instar larvae. Five larvae per tube were allowed to eat for 24 hours using 10 tubes per treatment, set up as in the last section (Results 1. (ii) (f) (A)). The frass collected was suspended in 5 ml of 0.02% sodium hydroxide in distilled water.

The results of this test are given in Table 10. Larvae ate less of the diet to which sugar was added compared to the diet with no addition of sugar. A one way analysis of variance revealed a significant difference at the 5% level between the 2 variates.

TABLE 10

Effect of varying the sugar content of the diet on the quanity consumed by second-instar *P. brassicae* larvae during 24 hours. Temperature 20 ± 1° C.

Sucrese addition to the diet (% w/w)	No. of replicates	Transmission (%)			Mean Consumption of diet mg.
		Mean	S.D.	95% Confidence interval of the mean	
0	10	58.49	4.20	55.48 - 61.5	13.7
3.3	10	63.68	5.56	61.99 - 65.37	11.6
6.6	10	62.58	5.21	58.85 - 66.31	11.9

2. Bioassay.

(i) Dosage-Mortality of Second-, Third- and Fourth-Instar Larvae.

The dosage-mortality of second-instar virus-free larvae was assessed by using phenol red to estimate the actual number of capsules ingested by individual larvae. Two concentrations of virus, 3.2×10^3 and 6.4×10^3 capsules 1 µl in both 0.5% phenol red and 0.2% Teepol L were used to treat cabbage leaf strips measuring 0.7 x 8 cm. Twenty µl of suspension were spread with a fine glass rod over the upper surface of each strip.

Racks were constructed of 0.6 cm thick perspex measuring 13 x 13 cm, holding 7 rows of 7 plastic specimen tubes 1.2 cm outer diameter by 6.2 cm long. Newly moulted second-instar larvae were then placed individually in 98 tubes and each strip of leaf, once dried, was carefully placed over the mouths of a row of 7 tubes. The mouth of each tube was only three-quarters covered with the leaf so as to provide adequate ventilation for the larvae. The leaf strips were held in position by placing one 13 by 13 cm glass plate over them and one under the bases of the tubes the two plates being held together with rubber bands (Figs. 7 and 8).

After 24 hours the larvae were removed and maintained individually on standard diet and the mortality was checked daily until pupation.

Figs. 7, 8

Exposed and assembled racks of tubes used for the determination of the LD_{50} of second-instar larvae with phenol red.

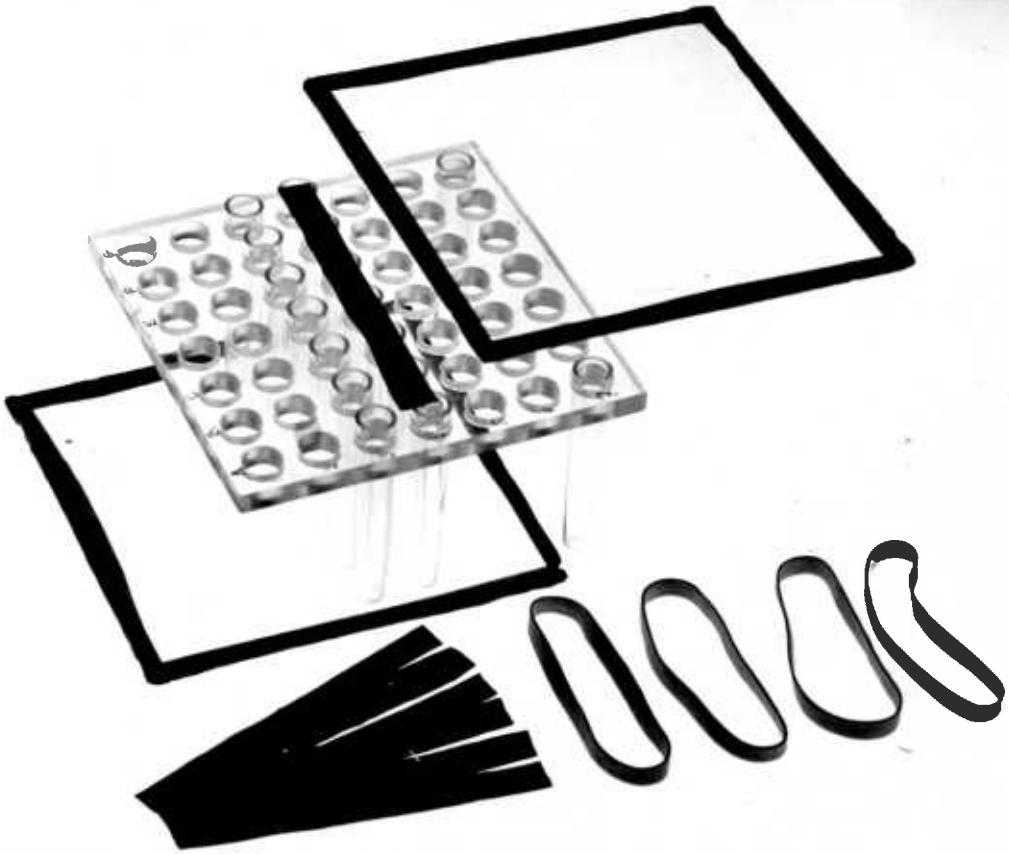


Fig. 7

Fig. 8

The frass collected in each specimen tube, containing the phenol red, was suspended in 1 ml of 0.02% Na OH and the optical density of each sample was measured in 1 ml microcuvettes at 560 nm in a Pye SP 30 Spectrophotometer.

The virus dose ingested by each larva was estimated assuming that the amount of phenol red recovered from the frass was directly proportional to the number of capsules, and was based on :

$$\text{No. of capsules ingested per larva for the first concentration} = \text{Optical density of the frass} \times 0.00059 \times 10 \times \frac{3200}{0.005}$$

$$= \text{Optical density of the frass} \times 3776$$

$$\text{For the second virus concentration } (6.4 \times 10^3 \text{ capsules } \mu l) = \text{Optical density of the frass} \times 7552$$

Where : optical density of the frass x 0.00059 x 10 gives the conversion factor for calculating the mgs of phenol red in 1ml of frass suspension. By multiplying the results by $\frac{3200}{0.005}$ the number of capsules per unit weight of phenol red can be calculated.

No corrections were made for the amount of phenol red not recovered from the frass, which was estimated to be less than 5% for individual larvae, as suggested from results of the recovery test in section 1. (ii) (b).

The larvae dosed with virus at the two concentrations were pooled and then split into four groups according to the number of capsules ingested. The average number of capsules ingested per larva in each group was calculated. The dosage mortality response was analysed by probit methods (Finney 1971) in a computer programme and the line is shown in Fig. 9.

The LD_{50} for second-instar larvae was 1.1×10^3 capsules with a slope of 1.14 and the χ^2 test for heterogeneity was not significant (Table 11). As few as 68 capsules killed one larva, however 2 larvae survived after ingesting over 1800 capsules.

From the 98 larvae set up, 2 died 24 hours after dosing from unknown causes, and 2 others were lost during handling.

The dosage-mortality response of each assay with third- and fourth-instar larvae, was independently analysed (see Tables 25 and 26 in Addendum) using the same probit method, as for second-instar larvae. A semi-weighted mean of the combined LD_{50}s was calculated for each instar taking into account the heterogeneity between LD_{50}s.

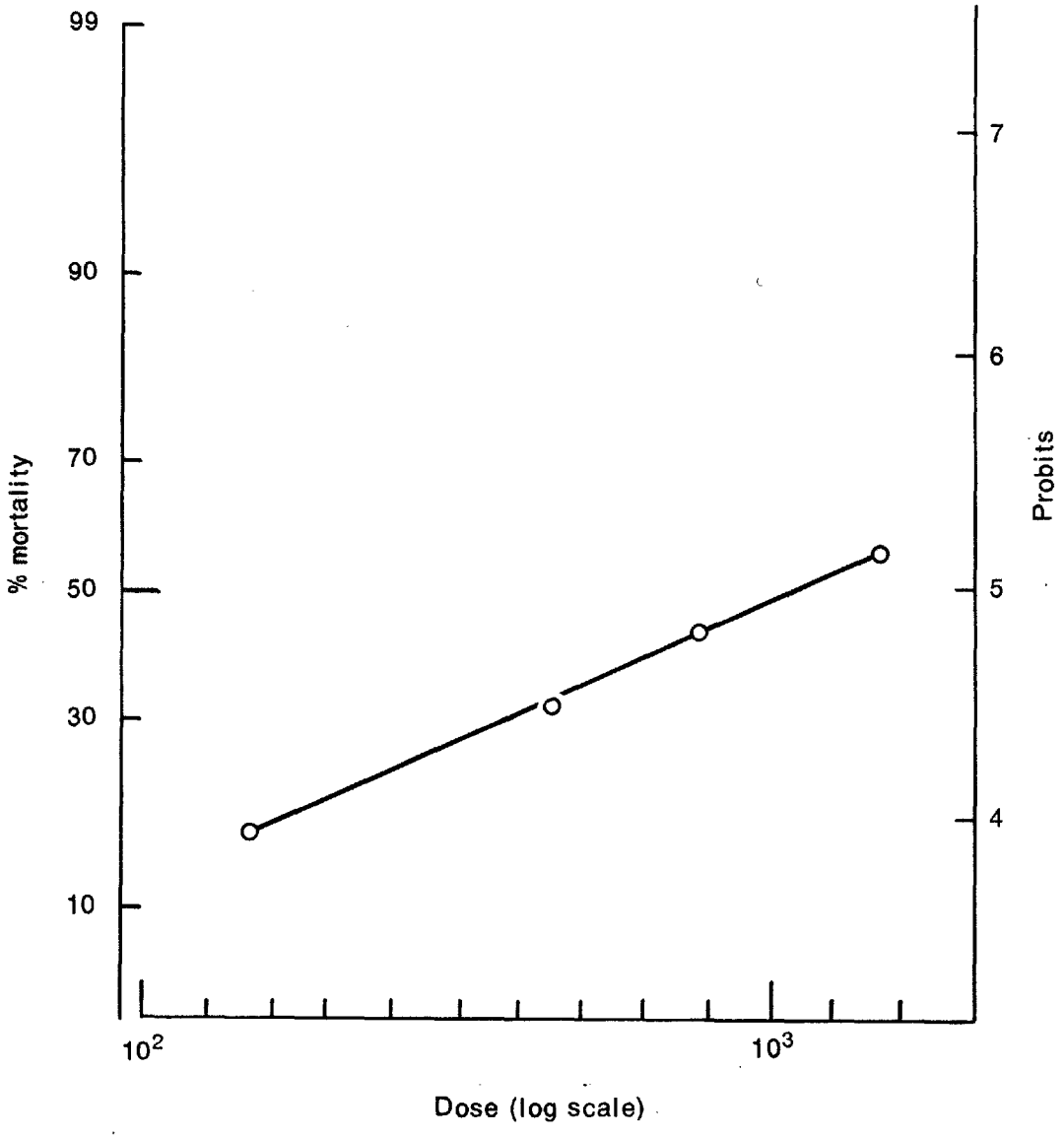


Fig. 9

Dosage-mortality response of 2nd instar larvae estimated by the phenol red method.

TABLE 11

Analysis of dosage-mortality for second-instar virus-free larvae estimated by the phenol red method.

LD_{50} capsules/larva	SE LD_{50}	95% Fiducial limits for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 for heterogeneity	No. larvae in test
1.1×10^3	1.4	6.4×10^2 - 8.0×10^3	1.14	0.40	0.010	94

SE = standard error

The mortality response of each test is given in Fig. 10 for third-instar and in Fig. 11 for fourth-instar larvae of the virus-free stock. The dosage-mortality lines were fitted using the semi-weighted LD_{50} and the slopes given in Tables 12 and 13.

Similar slopes (1.62 and 1.50) of the dosage-mortality lines were obtained for both instars but the number of capsules required for an LD_{50} was approximately 25 times higher for fourth-instar with no overlap of the confidence interval for LD_{50}s. In most tests a small number of larvae died one or two days after dosing, probably because of handling effects, and were not included in the results.

The time-mortality studies suggested a bimodal response in both instars of the virus-free stock with the slope changing after 10 days in third-instar larvae and after nine in fourth-instar larvae. The first deaths in third-instar larvae occurred in six days for the higher dosages and 7 days for the lowest dosage. Fourth-instar larvae started to die after 6 days with the highest dosage, after seven days for the following two, lower dosages, and after ten days with the lowest dose tried (Figs. 12 and 13).

The LT_{50} values for third-instar larvae were 8.9 days for 6.4×10^6 capsules/larva and 6.8 days for 6.4×10^7 and in fourth-instar larvae they were 9.7 days

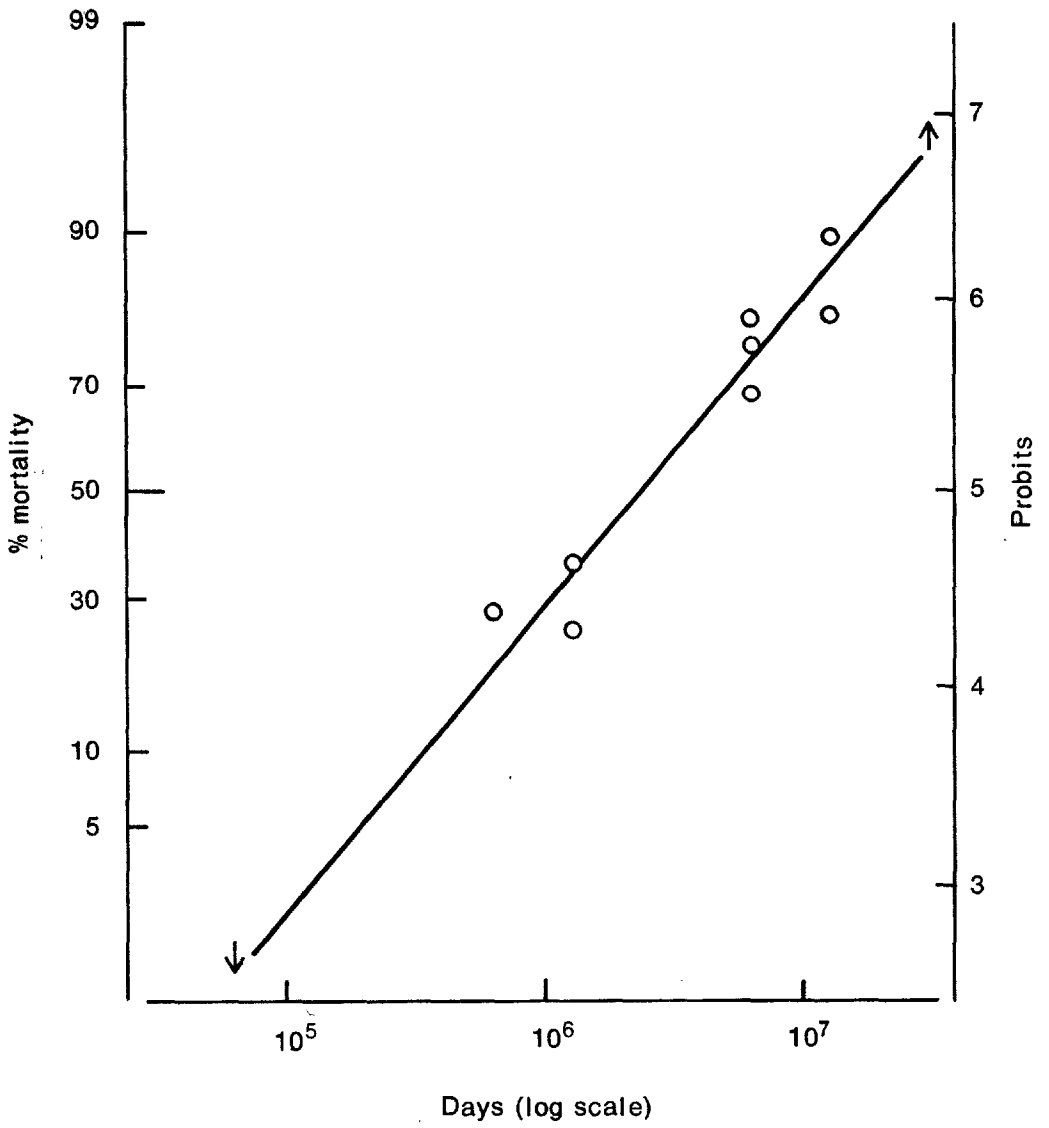


Fig. 10

Dosage-mortality response of 3rd instar larvae. Arrows indicate 0 or 100% mortality.

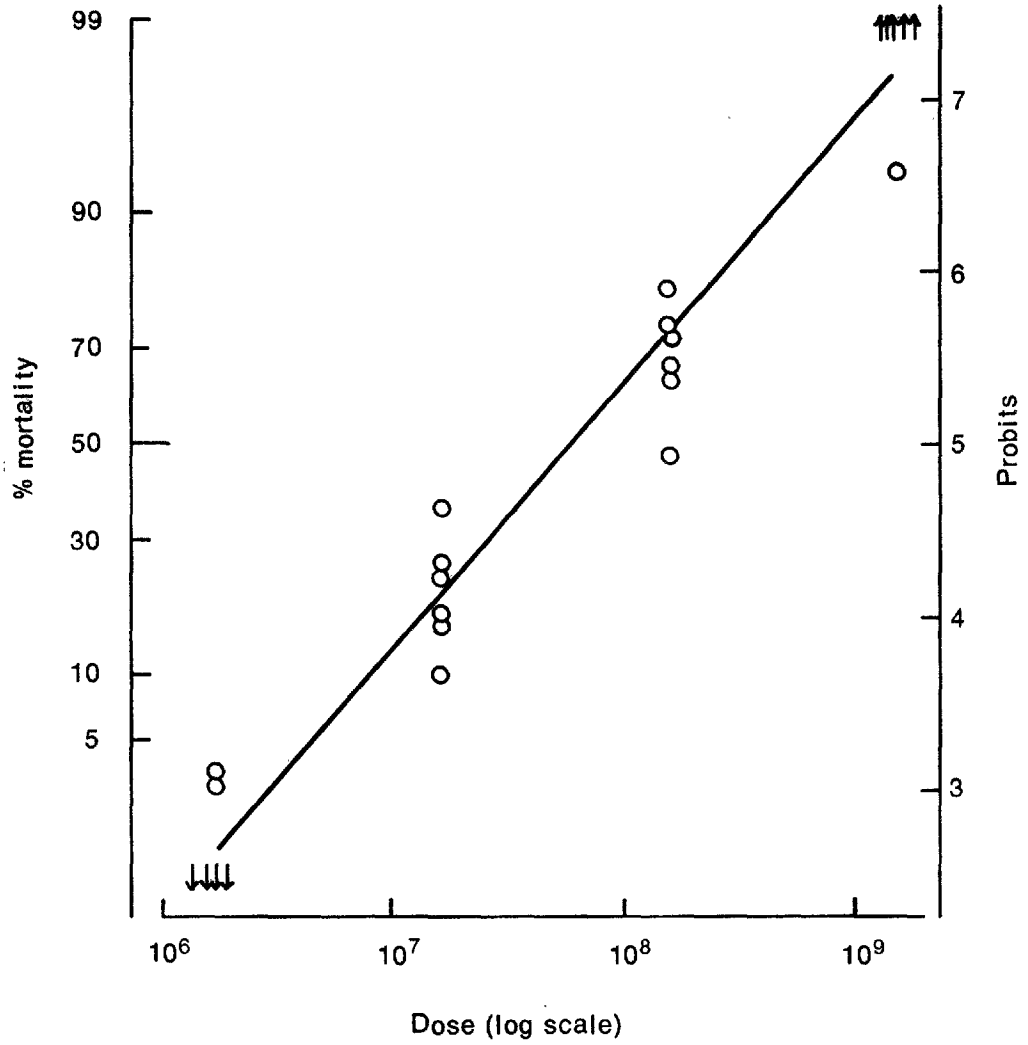


Fig. 11

Dose-mortality response of 4th instar larvae. Arrows indicate 0 or 100% mortality in replicated experiments.

TABLE 12

Combination of the three probit lines of third-instar larvae of P. brassicae.

Semi-weighted LD_{50} capsules/larva	SE [a] LD_{50}	95% confidence interval for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 heterogeneity for the slope	Total No. of larvae
2.4×10^6	1.21×10^3	$1.6 \times 10^6 - 3.5 \times 10^6$	1.62	0.15	0.03	436

[a] The SE of the LD_{50} was based on the antilog of the SE for the log LD_{50}s

TABLE 13

Combination of the six probit lines of fourth-instar larvae of P. brassicae.

Semi-weighted LD_{50} capsules/larva	SE [a] LD_{50}	95% confidence interval for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 heterogeneity for the slope	total No. of larvae
6.1×10^7	1.15×10^5	$4.8 \times 10^7 - 7.7 \times 10^7$	1.50	0.10	0.035	705

[a] see footnote Table 12

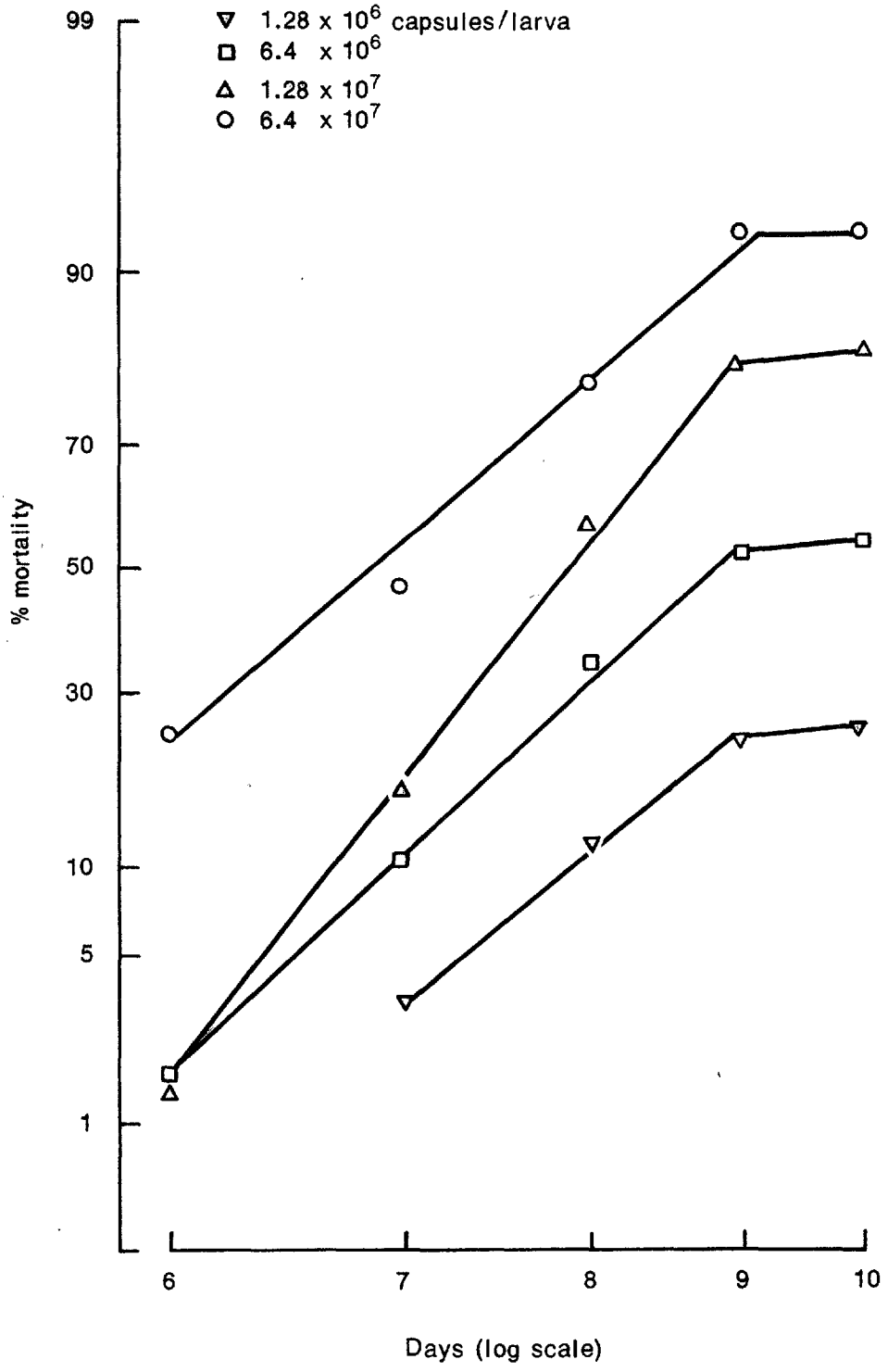


Fig. 12

Time mortality response of 3rd instar larvae to different doses of virus.

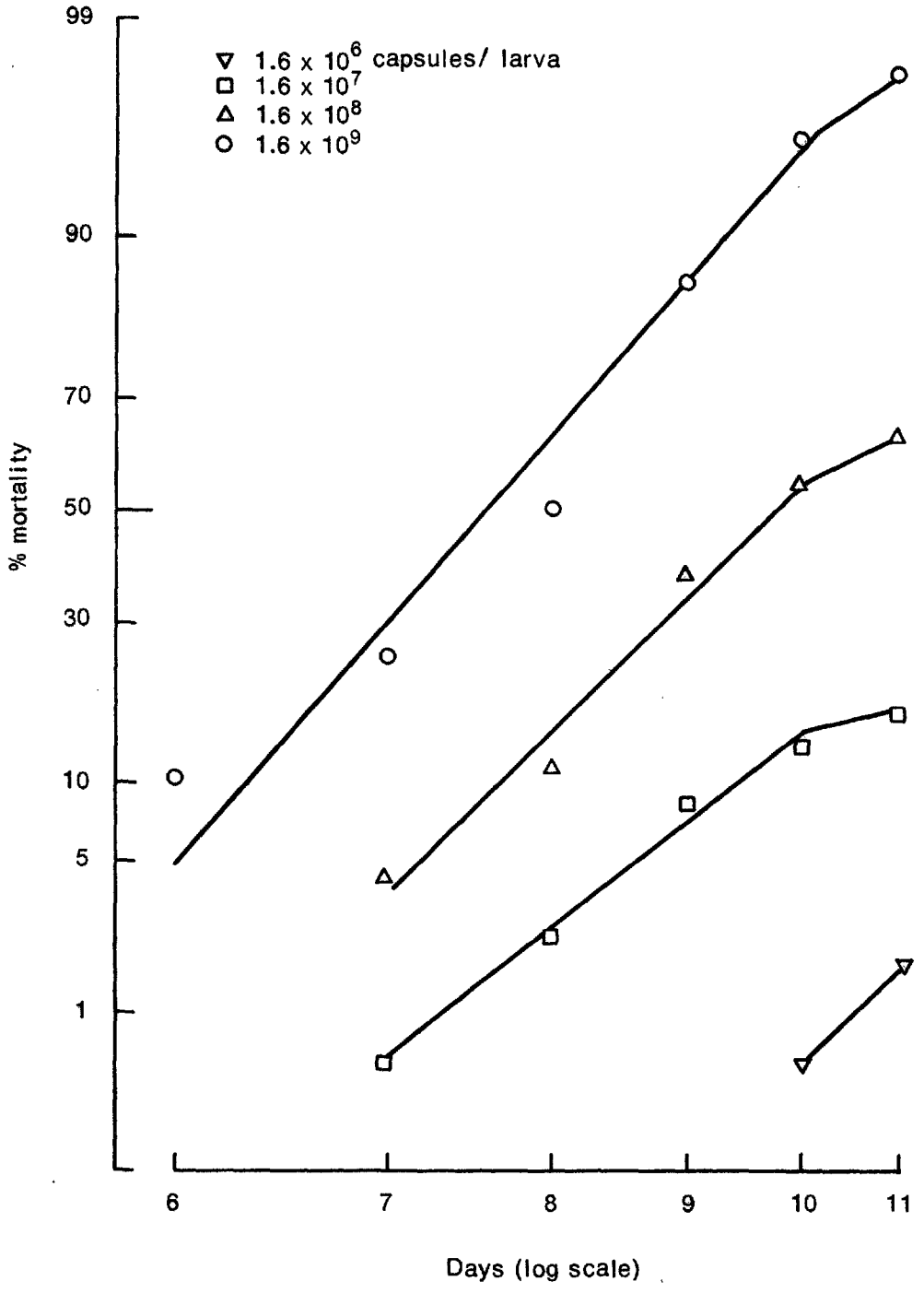


Fig. 1 3

Time-mortality response of 4th instar larvae to different doses of virus.

at 1.6×10^8 capsules/larva and 7.5 days at 1.6×10^9 capsules/larva . The time-mortality lines were fitted by eye. When third- and fourth-instar larvae were fed similar dosages (1.28×10^7 and 1.6×10^7 respectively) the first mortality occurred 1 day earlier (on the 6th day) for the third-instar larvae.

(ii) Comparative Susceptibility to Granulosis Virus of four stocks of P. brassicae.

The susceptibility of second-instar larvae of four stocks of P. brassicae to granulosis virus is given in Table 14. Considerable variations between tests on each stock were observed as indicated by the large value of the standard error (SE) of the average mortality in each case.

At the higher dosage of 3.2×10^5 capsules per tube the cheiranthi stock was the most susceptible followed, in decreasing order, by the French, Dutch and virus-free stocks. Mortalities obtained with the lower dosages also indicated that the cheiranthi larvae were most susceptible but the susceptibility of other three stocks was similar. The first deaths (about 1%) occurred 5 days after dosing with these three stocks but, with the virus-free larvae mortality started with 5.5% deaths on the 6th day.

Parallel dosage-mortality bioassays with fourth-instar larvae of the 4 stocks and granulosis virus showed also the higher susceptibility of the cheiranthi stock and

TABLE 14

Susceptibility of second-instar larvae of four stocks of P. brassicae to two concentrations of granulosis virus.

Stock	Dose, capsules per tube	Average mortality (%)	Standard error of the average	No. of larvae used
cheiranthi	3.2×10^5	79.47	4.59	653
	3.2×10^3	5.57	2.76	646
French	3.2×10^5	70.30	8.34	1007
	3.2×10^3	1.58	0.25	1009
Dutch	3.2×10^5	63.63	4.62	781
	3.2×10^3	1.95	0.35	766
Virus-free	3.2×10^5	46.8	5.23	1600
	3.2×10^3	1.28	0.42	768

comparisons based on the LD_{50} dose showed that the fourth-instar cheiranthi larvae were 12 times more susceptible than the same instar of the virus-free larvae.

The Dutch and French stocks showed very similar susceptibilities the French being slightly more resistant as shown in Table 15. However the 95% fiducial limits of the LD_{50}s show almost complete overlap. Small differences in the slopes of the dosage-mortality lines were observed when compared to the virus-free slope (1.50) drawn as a reference in Fig. 14. The French stock showed the least steep slope of all the four stocks. For greater clarity the time-mortality graphs for only one dosage (3.2×10^6 capsules/larva) are shown in Fig. 15. The cheiranthi Dutch and French stocks showed similar slopes with a truncation on the 9th day after dosing, compared to the 10th day for the virus-free stock. Most of the infected larvae had died by this time.

At similar levels of total mortalities the virus-free larvae started to die 7 days after being dosed with 1.6×10^7 capsules/larva whereas the other three stocks started to die on the 6th day after being dosed with 3.2×10^6 capsules/larva.

TABLE 15

Probit analysis of fourth-instar larvae from three stocks of P. brassicae.

Stock	LD_{50} capsules/larva	SE LD_{50}	95% fiducial limits for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 for heterogeneity	No. of larvae
cheiranthi	5.0×10^6	1.6×10^5	1.2×10^6 - 1.9×10^7	1.44	0.10	54.7	701
French	1.0×10^7	1.3×10^5	6.1×10^6 - 1.8×10^7	1.25	0.08	11.9	669
Dutch	8.9×10^6	1.2×10^5	5.7×10^6 - 1.5×10^7	1.59	0.09	12.5	726

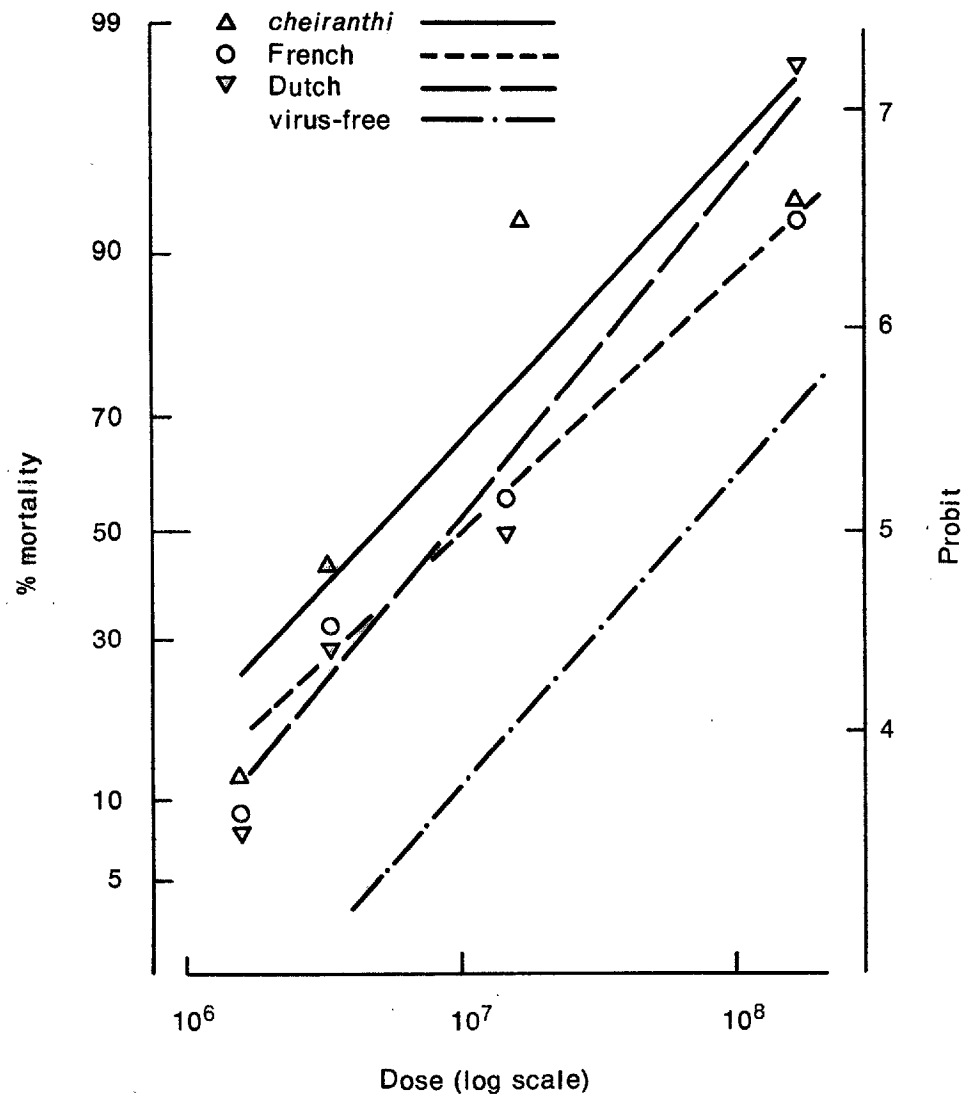


Fig. 14

Dosage-mortality response of 4th instar larvae of four stocks of *P. brassicae*.

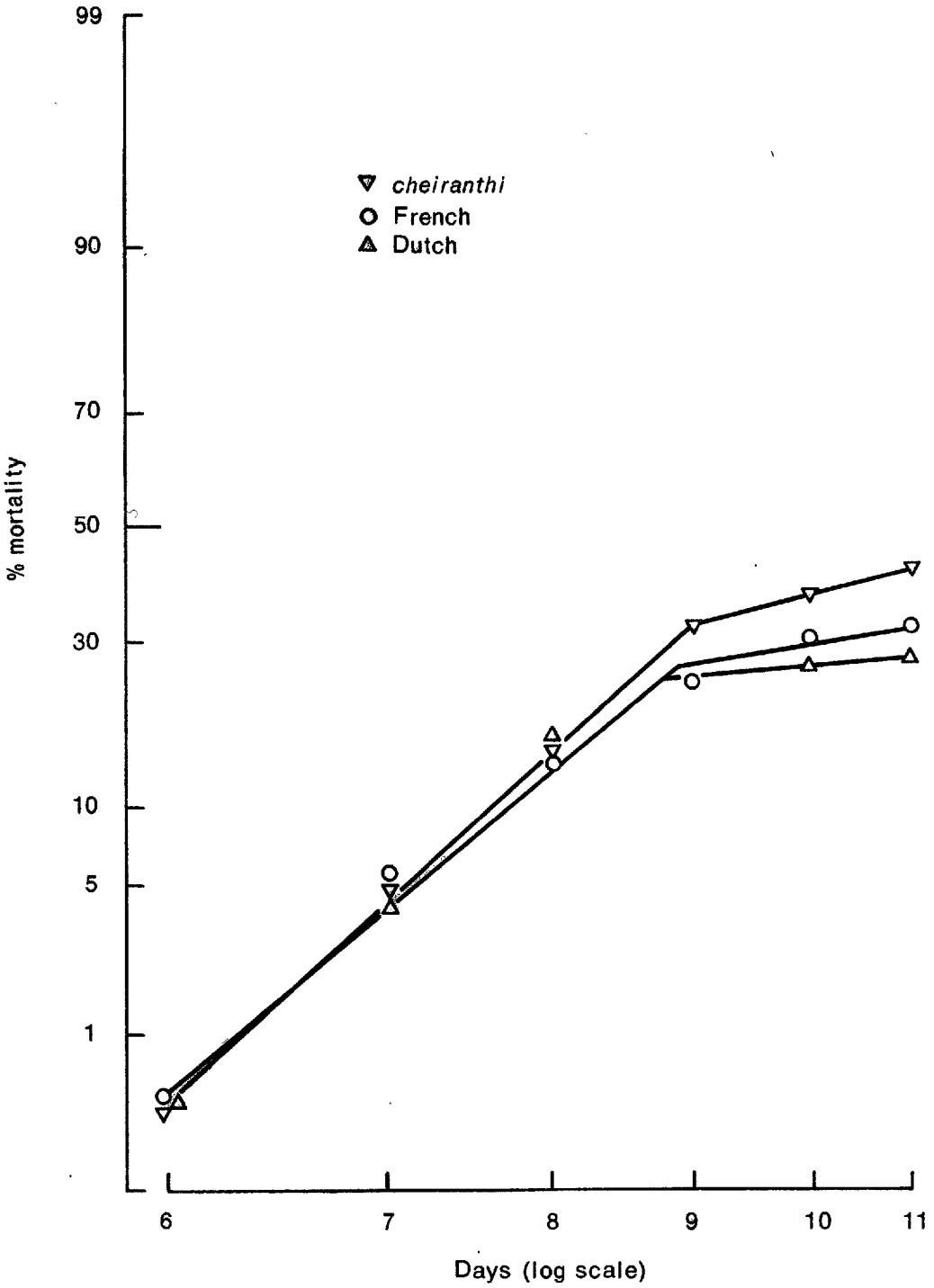


Fig. 15

Time-mortality response of 4th instar larvae of three stocks of *P. brassicae* to a dose of 3.2 x 10^6 capsules per larva.

(iii) Effects of Different Treatments on Virus Viability.

(a) Effect of Gut Fluid and Buffers.

The loss of infectivity of granulosis virus which was subjected for 1 minute to a range of pHs similar to that encountered in the mid-gut of P. brassicae larvae is presented on Fig. 16. The infectivity was bioassayed with second-instar larvae.

The heat treated gut fluid at pH 9.8 or below had a slight inactivating effect which decreased as the alkalinity of the treated gut fluid was reduced. A sharp decline in infectivity of the virus was observed at pH 9.9 to 10.0 (Fig. 16). Gut fluid seemed to have a slightly more pronounced inactivating effect than either buffer or heat treated gut fluid at this pH (Table 16).

The loss of viability with alkalinity in two buffer solutions was compared with the dissolution of the capsules using optical density measurements. Sodium carbonate-sodium bicarbonate and borate buffers both at 0.1 M were prepared (Dawson et al., 1969). Fifty µl of granulosis virus containing 1.6×10^{10} capsules were added to 4 ml of the buffer in a plastic cuvette which was shaken and immediately placed in a Pye SP 30 UV Spectrophotometer and the optical density was recorded graphically at 350 nm, for 14 minutes.

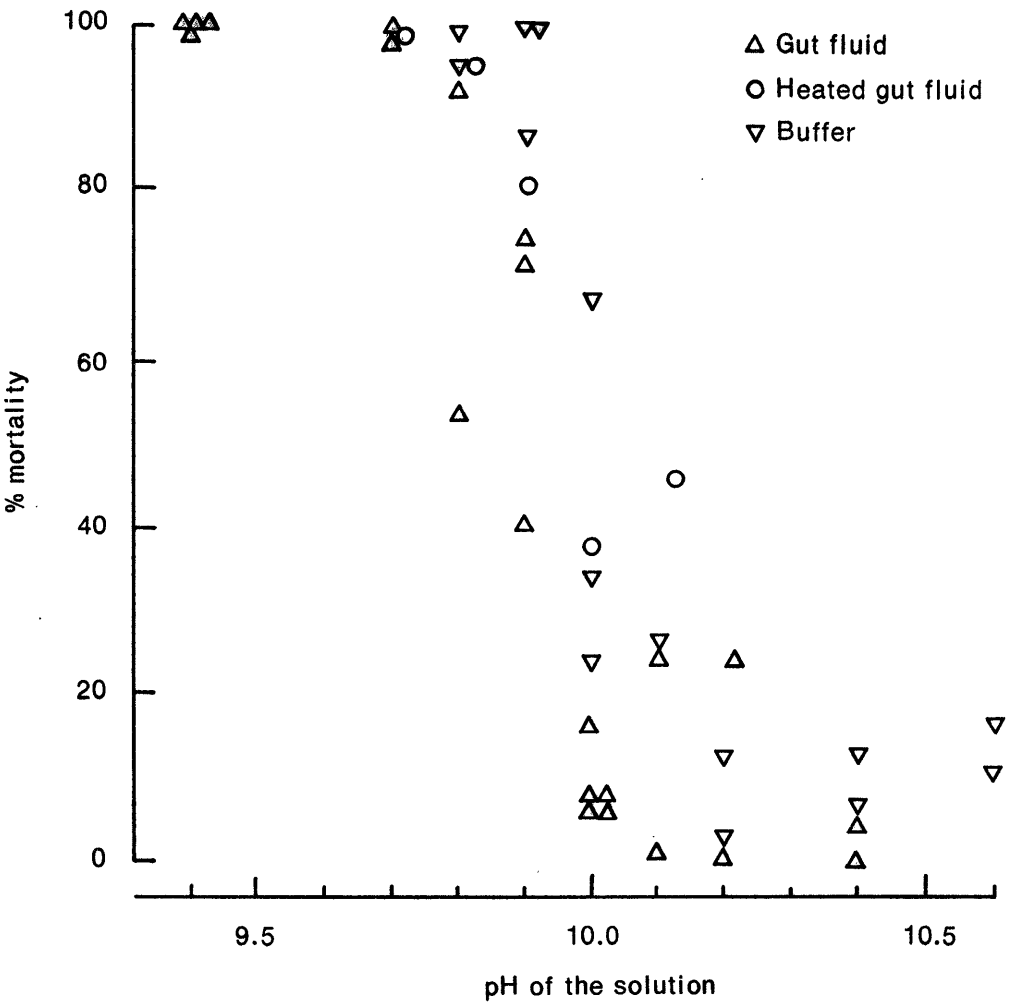


Fig. 16

Loss of viability of granulosis virus subjected to gut fluids and buffers of different pH for 1 minute and then bioassayed with 2nd instar virus-free larvae of *P. brassicae*.

TABLE 16

Granulosis virus viability after one minute treatment at different pHs expressed as average mortality of second-instar P. brassicae larvae.

pH	Gut fluid		Heat treated gut fluid		Buffer	
	Mortality (%)	No. of larvae	Mortality (%)	No. of larvae	Mortality (%)	No. of larvae
9.4	99.7	795	-	-	-	-
9.7	98.7	389	99.4	196	-	-
9.8	72.8	402	96.0	200	97.4	395
9.9	62.6	591	81.8	198	95.7	587
10.0	9.55	984	37.8	177	42.3	591
10.1	12.9	393	47.7	197	26.1	198
10.2	13.0	398	-	-	8.52	387
10.4	2.43	369	-	-	10.1	396
10.6	-	-	-	-	14.1	389

The dissolution curves obtained are shown in Fig. 17 and an average between the pH measurement before and after the dissolution is indicated for each curve. The dissolution rate of the capsules varied with the pH and the type of buffer. The carbonate buffer dissolved the capsules at pH 9.35 but at pH 9.45 the borate buffer had, apparently, no effect. The optical density of the virus suspension reached 0.180 after 1 minute with the carbonate buffer pH 9.80, and after 2 minutes with borate buffer at pH 9.85.

(b) Inactivation of Granulosis Virus in the Alimentary Canal of P. brassicae and of two other Lepidoptera.

Mature larvae of P. brassicae, Lacanobia oleracea and Spodoptera littoralis were used to investigate the inactivation of granulosis virus in the gut of 3 species. Two of the 3 species showed a distinct relationship between the pH of the alimentary canal and the inactivation of the virus when bioassayed with second-instar virus-free larvae.

The highest pH in the alimentary canal, which is about 10.3 is in the center mid-gut of P. brassicae and when the virus reaches this point most of the inactivation seems to take place, corresponds to the lowest average activity of the virus shown in Table 17.

In the three larvae of Lacanobia oleracea there was no corresponding rapid inactivation when the

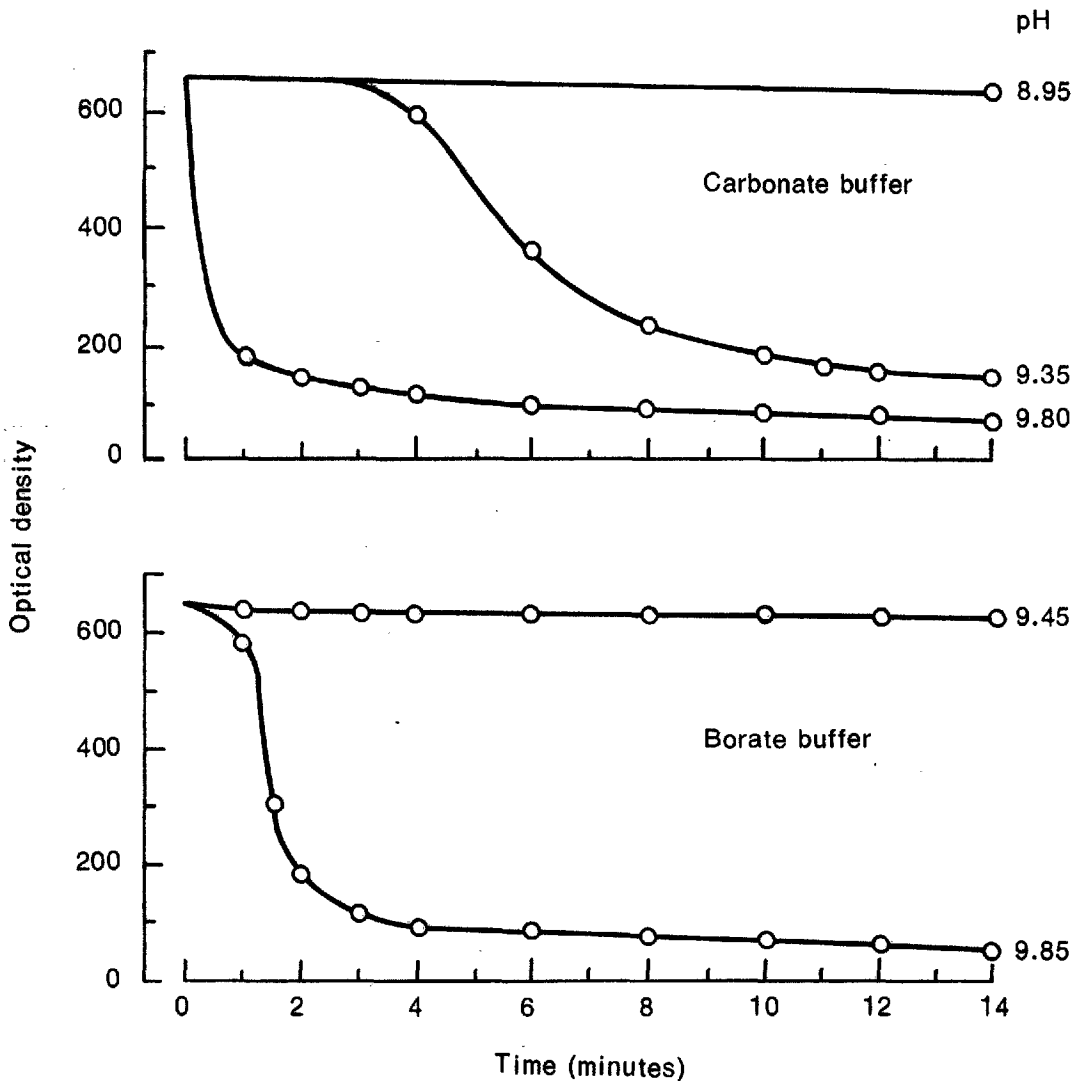


Fig. 17

Alkaline dissolution of granulosis virus by two buffers, measured by the change in optical density at 350 nm.

virus reached the mid-gut where again the pH was highest. The lowest average activity of the virus was found in the last section of the alimentary canal. Spodoptera littoralis showed the highest pH in the anterior mid-gut where viability was lost rapidly (Table 18). However for reasons which cannot be explained activity was again high in the center mid-gut.

Mortality (%)[a]

	Alimentary canal section				
Larva Number	Crop	Anterior mid-gut	Center mid-gut	Posterior mid-gut	Ileum-rectum
1	--	25	0	3.4	8.6
2	86.6	45.7	0	1.69	1.7
3	88.1	9.2	1.7	6.6	6.8
4	--	94.8	0	5.1	14.0
5	3.4	1.6	0	0	3.3
6	6.8	16.9	3.6	0	5.2
Average	46.2	32.2	0.88	2.79	6.6

(a) Expressed as percentage mortality based on 60 second-instar larvae divided into three tubes for each for 6 individuals assayed fifth-instar larvae.

Table 17

Inactivation of granulosis virus passing through the alimentary canal of P. brassicae.

Mortality (%)[a]

Larva Number	Lacanobia oleracea alimentary canal sections				
	Crop	Anterior mid-gut	Center mid-gut	Posterior mid-gut	Ileum-rectum
1	25.4	30.5	7.5	54.9	28.8
2	87.5	100	91.1	30.7	21.8
3	9.8	70.9	24.0	65.4	26.0
Average Mort-ality	40.9	67.13	40.86	50.3	25.5
Average pH	8.0	9.2	10.2	8.7	8.0

Mortality (%)[a]

Larva Number	Spodoptera littoralis alimentary canal sections				
	Crop	Anterior mid-gut	Center mid-gut	Posterior mid-gut	Ileum-rectum
1	84.9	8.8	80.7	67.5	19.1
2	7.5	7.1	66.6	7.4	56.0
Average Mort-ality	46.2	7.9	73.6	37.4	37.55
Average pH	8.5	10.08	9.4	8.7	8.0

(a) see footnote in Table 17.

Table 18

Mortality of second-instar P. brassicae larvae used to measure the inactivation of granulosis virus passing through the alimentary canal of Lacanobia oleracea and Spodoptera littoralis and the pH in various regions of the gut of these larvae.

(c) Electron Microscope Observations.

Parallel to the viability studies of the virus described in the last two sections, the dissolution of the capsules in the gut fluid and buffers, already described in section 4. (iii) was observed with the electron microscope. For this a standard granulosis virus suspension and one which had been inactivated by heat treatment (90°C for 5 min) were used. Fifty µl of gut fluid or buffers were mixed with 1.6×10^9 capsules and after 1 minute the dissolution was stopped by adding 5 µl of 1 M citric acid phosphate buffer (Grimstone and Skaer 1972). The preparation was negatively stained with potassium phosphotungstate or uranyl acetate and viewed through an AEI EM6B electron microscope at 70 to 80 KV.

The standard granulosis virus granulin (Martignoni 1957) dissolved in the gut fluid and buffers after one minute exposure at about pH 9.8 or higher varying greatly in each individual preparation. On several occasions after treating capsules with gut fluid at pH 10.2 for 30 seconds neither capsules or virions could be found suggesting that both had already been degraded.

Glycine/Na OH buffer (Sorensen-Walbaum) 0.1 M pH 10.1 dissolved the capsules less completely after one minute and, in general, more desintegrating capsules were found than with gut fluid preparations of similar pH.

Figs. 18 - 27

Electron micrographs of granulosis virus of *P. brassicae*.

Fig. 18

Purified preparation of the virus stained with potassium phosphotungstate (PTA) (x 30,000).

Fig. 19

As above showing abnormal forms stained with PTA (x 30,000).

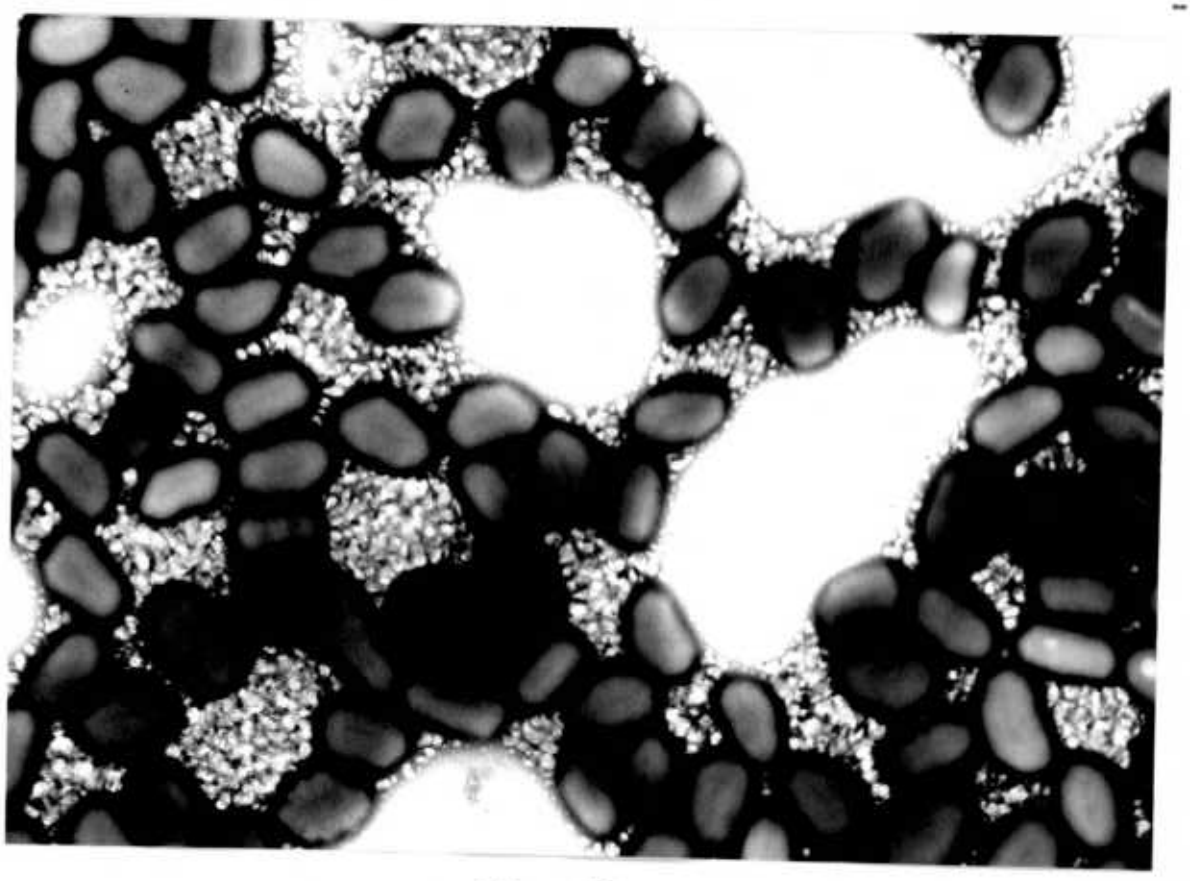


Fig. 18

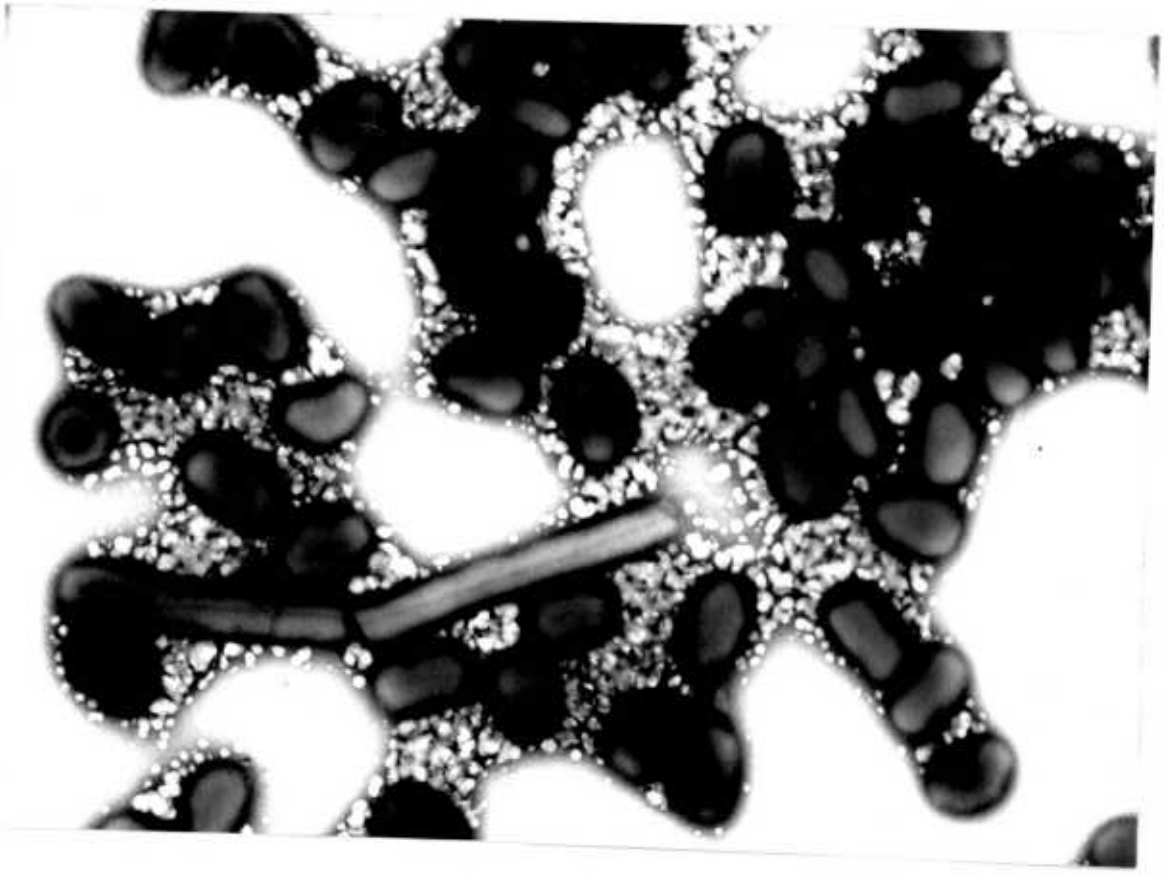


Fig. 19

Fig. 20

Capsules treated with buffer pH 10.1 for one minute (note swelling), stained with PTA (x 120,000).

Fig. 21

Same as above in a more disintegrated stage ; stained with uranyl acetate (x 120,000).

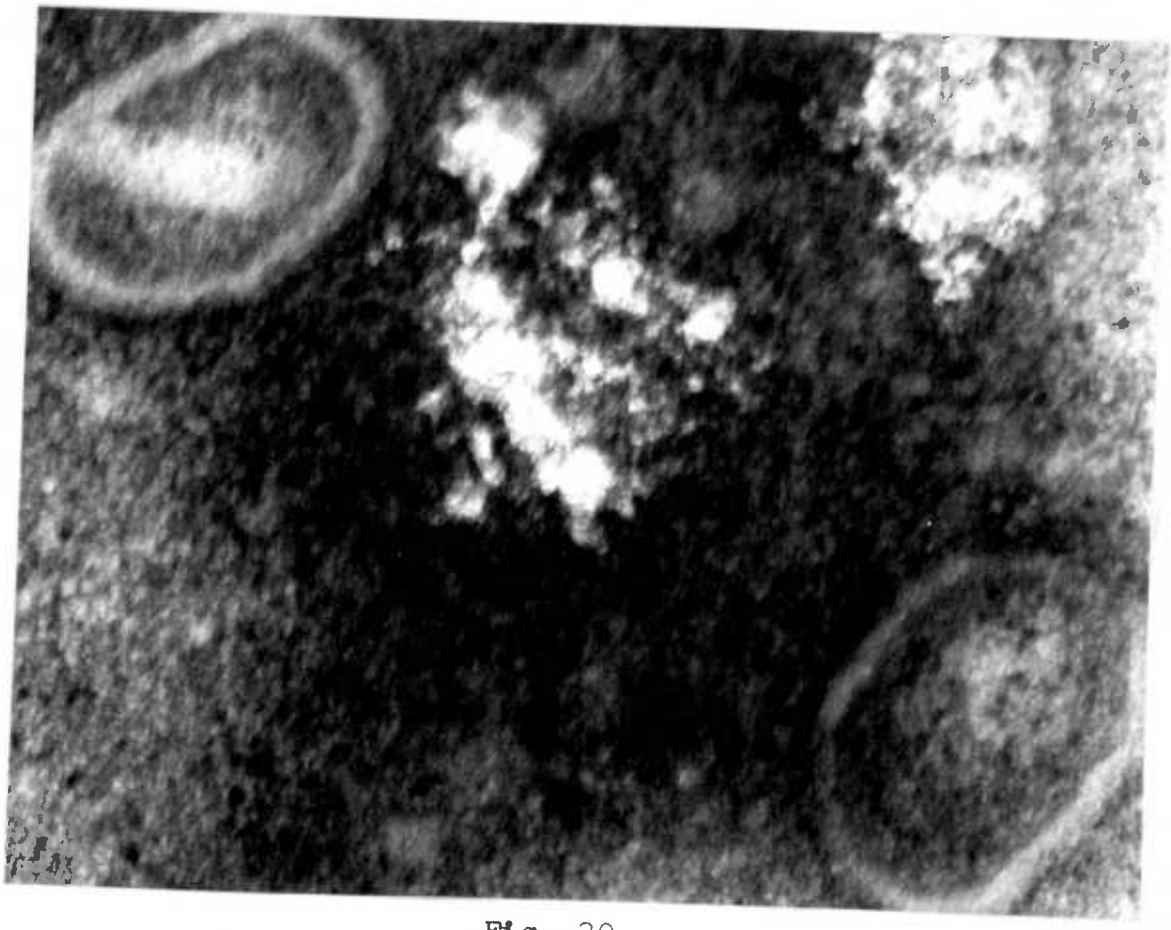


Fig. 20

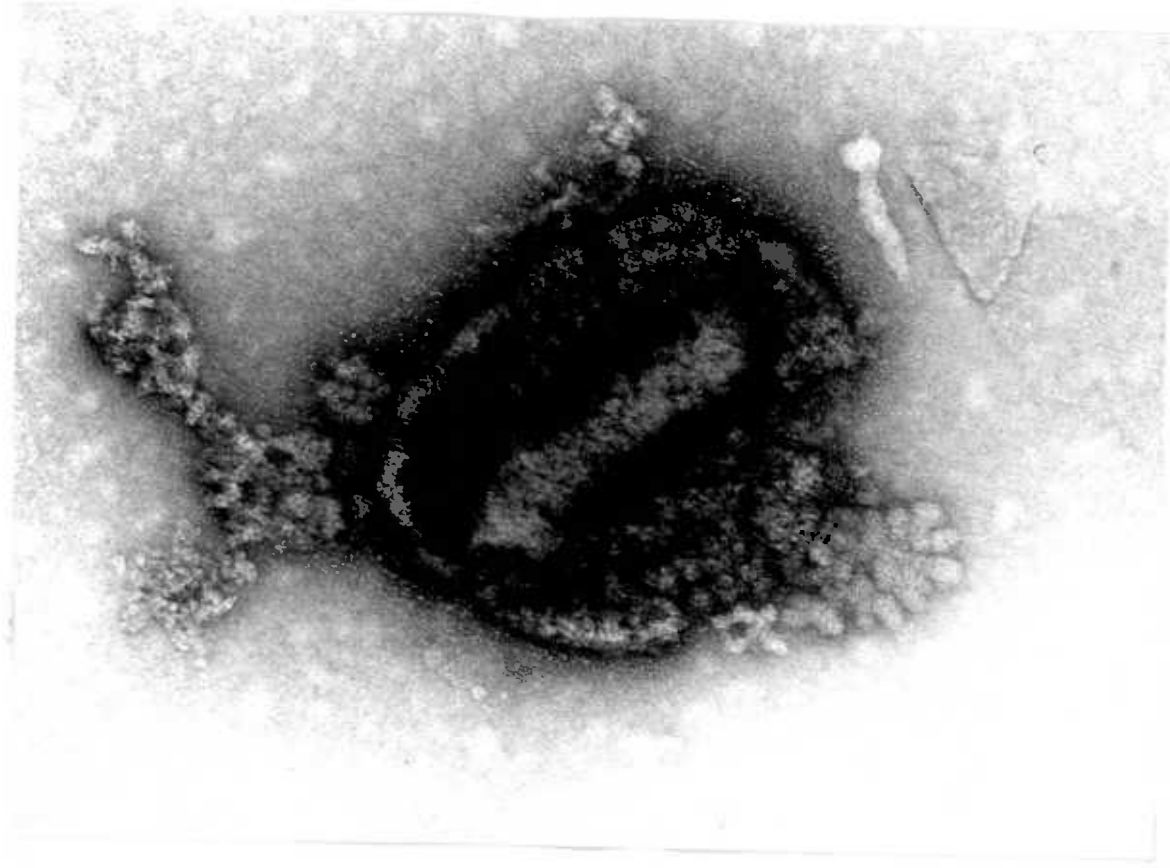


Fig. 21

Fig. 22

One of the rare capsules which survive exposure for two minutes to gut fluid from starved fifth-instar larvae, showing the virion almost free ; stained with PTA (x 120,000).

Fig. 23

Heat treated capsules exposed for one minute to gut fluid pH 10.3, showing dark area around the virion ; stained with PTA (x 120,000).

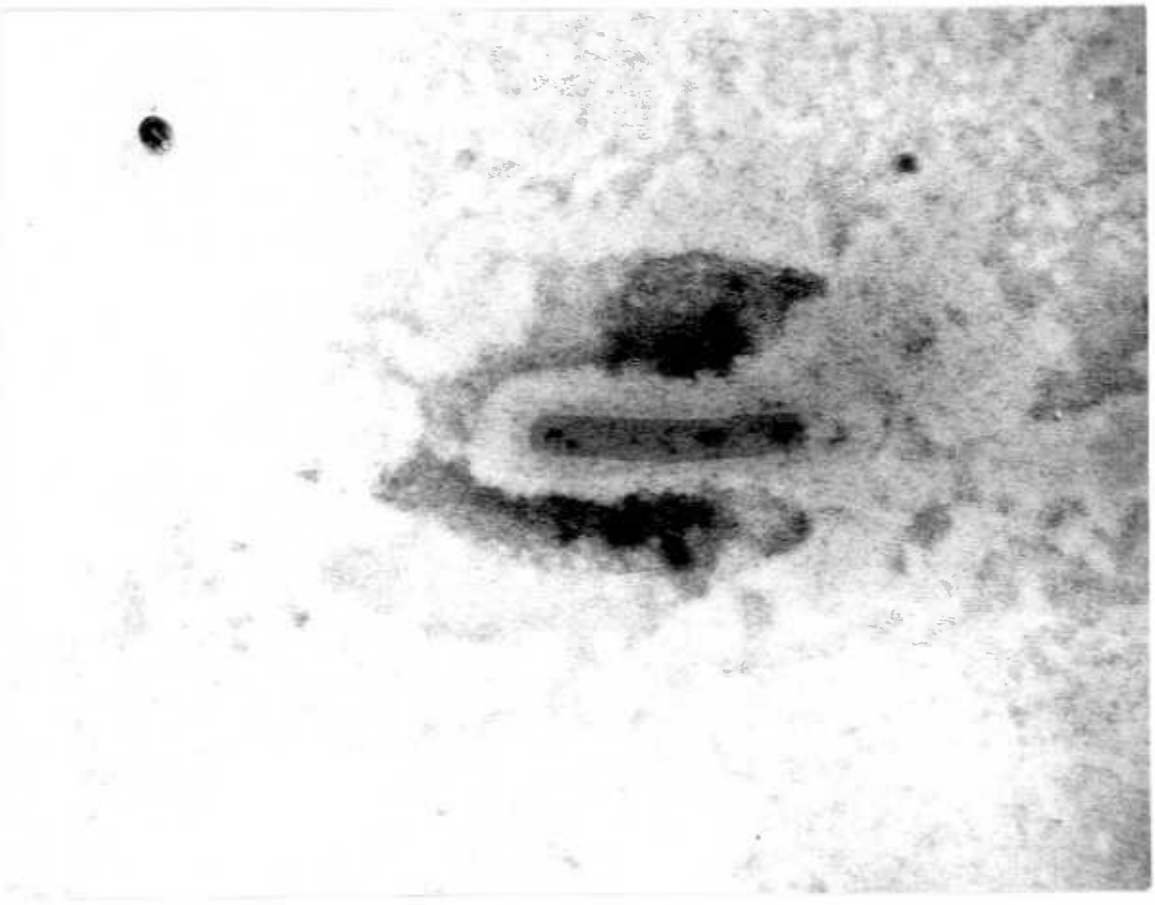


Fig. 22

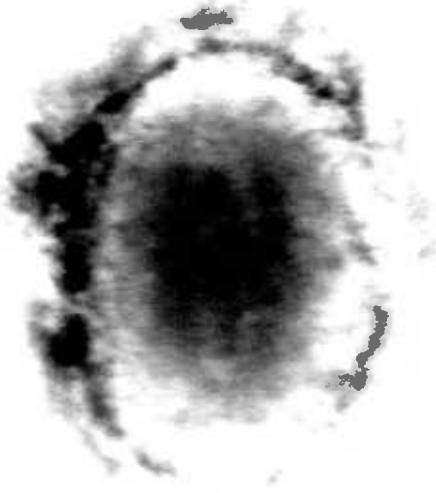


Fig. 23

Fig. 24

Heat treated capsules exposed for one minute to gut fluid pH 10.3 showing comparatively little disruption and dark area around the virion ; stained with PTA (x 75,000).

Fig. 25

As above showing a darker area on one end of the virion ; stained with PTA (x 75,000)

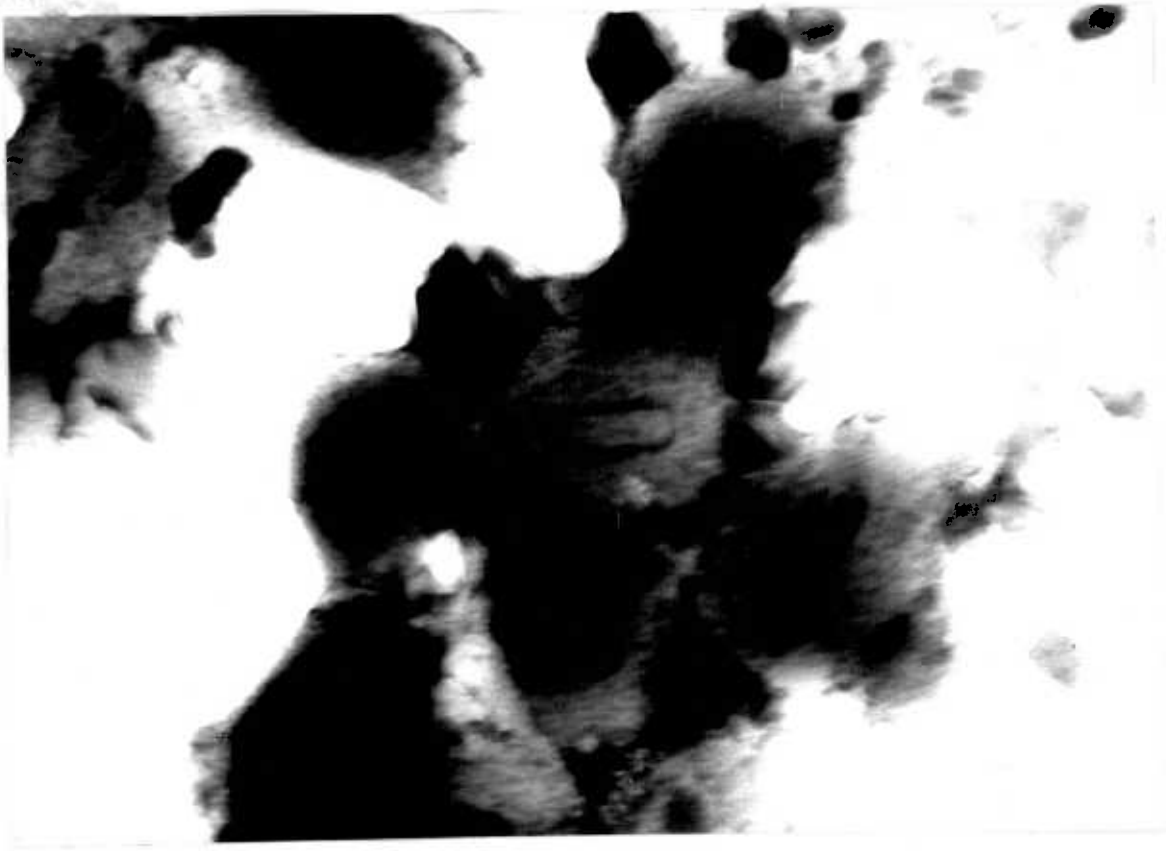


Fig. 24

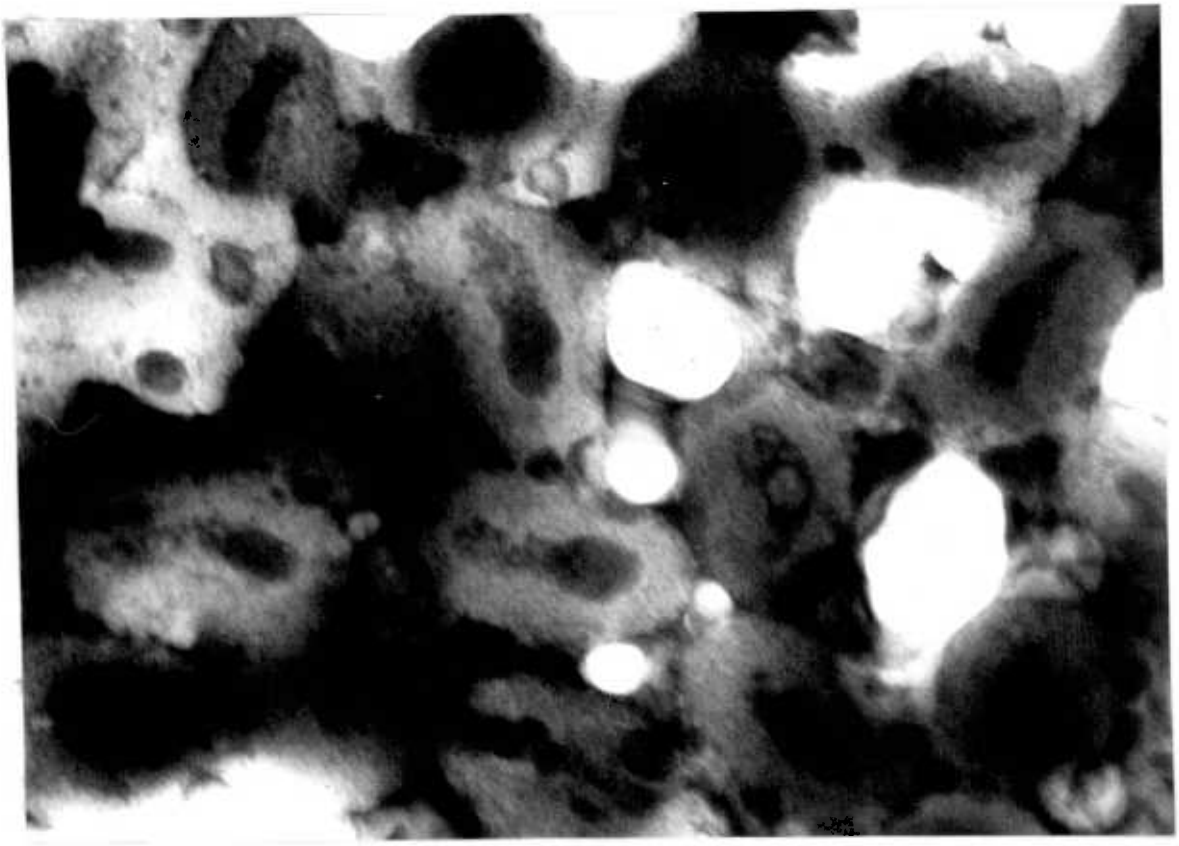


Fig. 25

Fig. 26

Clump of capsules exposed to buffer pH 10.1 for one minute ; note large number of virions ; stained with uranyl acetate. (x 30,000)

Fig. 27

As above, at higher magnification (x 60,000).

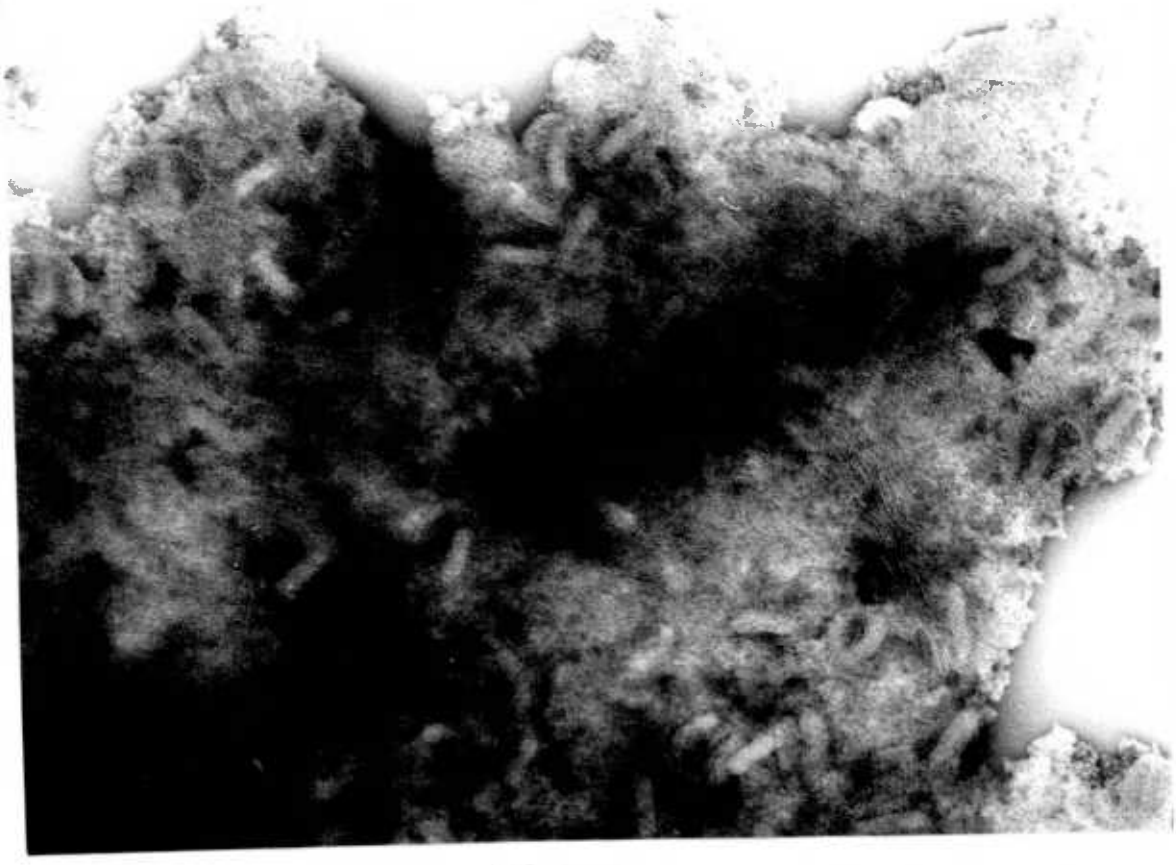


Fig. 26

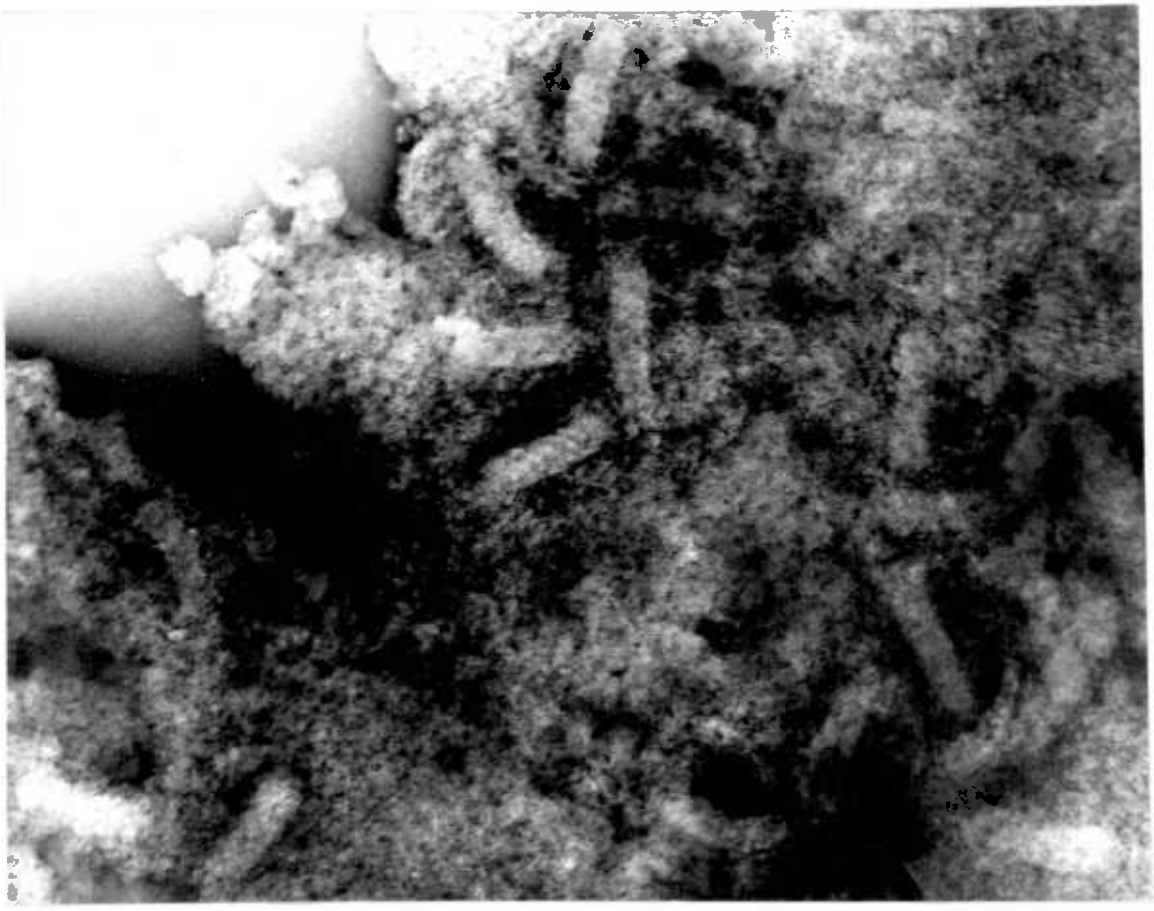


Fig. 27

The following morphological changes of the virus probably occur in alkaline conditions of buffer and gut fluid ; the capsule swells up, (Fig. 20), becomes more electron transparent and rounded and the granulin dissolves progressively from the inside, (Fig. 21), releasing the virion (Fig. 22).

The heat-treated capsules, subjected to gut fluid, showed, in some preparations, a dark area corresponding to the virion, as shown in Figs. 23, 24 and 25 and dissolved at a much slower rate in gut juice or buffer than untreated capsules.

(iv) Action of Diet Additives on Mortalities of Larvae fed with Granulosis Virus.

(a) Feeding Virus on Acidified Diet.

Second-instar larvae were fed on the acidified diet only during the 24 hours period in which they were dosed with the virus. Before and afterwards they were fed on standard diet. The influence of the pH of the diet surface was investigated because it was assumed that the acidity of the diet might affect the buffering system in the gut environment, possibly decreasing the mid-gut pH, in which the virus is disrupted and infects the larvae.

As the diet pH was lowered a higher overall mortality was obtained indicating an inverse relationship (Fig. 28). The correlation coefficient calculated from

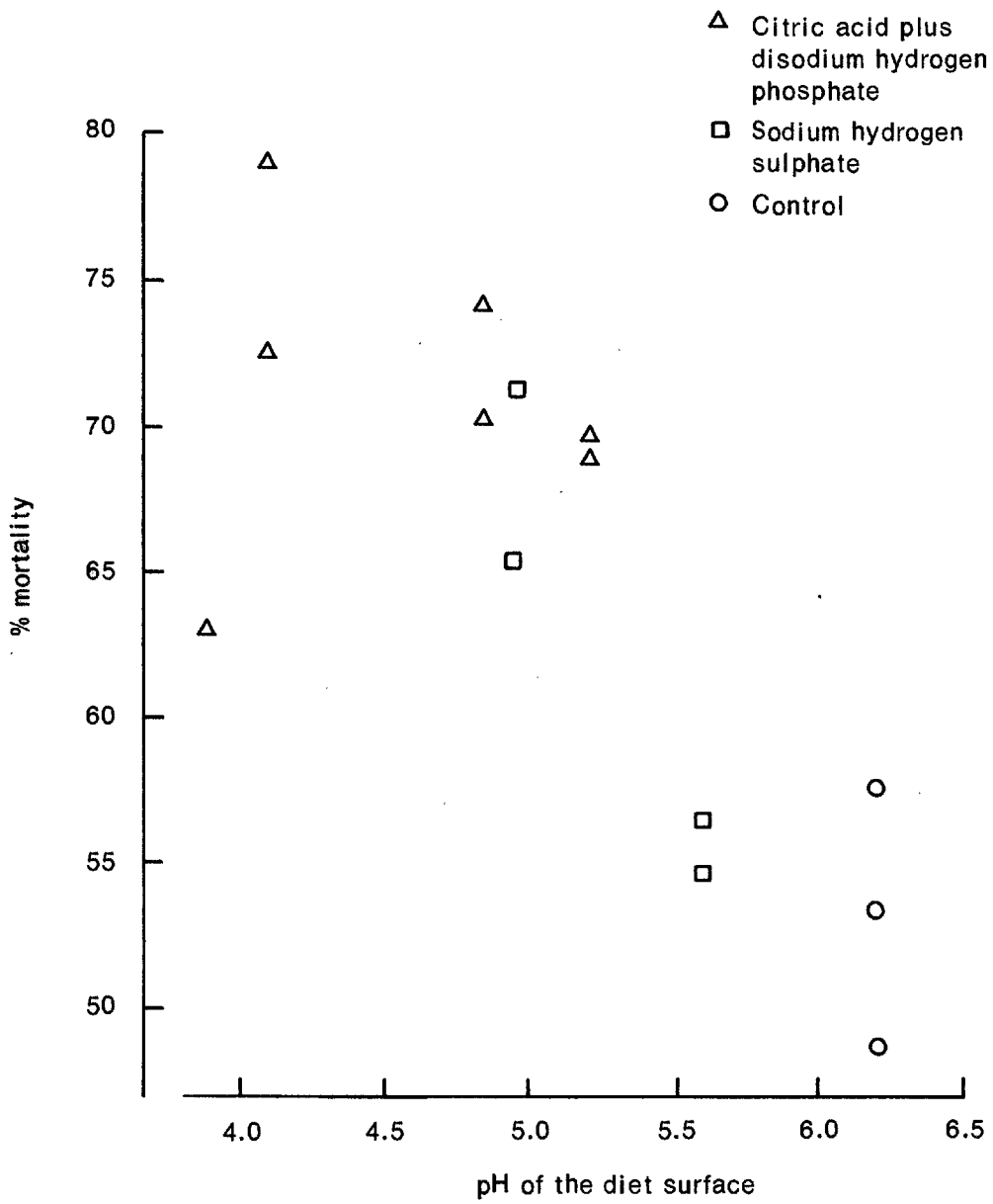


Fig. 28

Mortality of 2nd instar virus-free larvae dosed on acidified diet.

this data was r = 0.84, significant at P = 0.01. A total of 2136 second-instar larvae were used in this test, 235 of these maintained as controls and dosed with virus on standard pH (6.2) diet.

The influence of the acidification on diet consumption was measured with the phenol red method outlined in Results 1.(ii)(f). Three concentrations of citric acid plus $Na_2 HPO_4 . 2H_2O$ were added to the diet surface as mentioned previously and untreated controls were also kept. Each treatment consisted of 8 tubes with 20 newly moulted second-instar larvae per tube. The amounts of acidified diets consumed were similar to the control, except for the lowest ie. pH 3.9, of which 34.4% less was consumed.

(b) Effect of Oxalic Acid.

(A) On the Virus.

Oxalic acid solutions and virus suspensions were mixed and allowed to stand in darkness at $20^{\circ} \pm 1^{\circ}C$ for 24 hours. The virus was then bioassayed as previously described on formalin-free diet.

The results (Table 19) show that with a 24 hours exposure even at the lowest concentration tested, oxalic acid substantially inactivated the virus. No toxic effects were observed on the larvae from the oxalic acid feeding even at the highest concentration used.

Concentration of oxalic acid in the virus suspension (%) w/v	No. of deaths		No. of larvae treated
	Unidentified	Virus	
0.1	1	1	200
1	2	0	200
5	0	0	200
10	0	0	200
Control	0	48	192

Table 19

Inactivation effect of oxalic acid on granulosis virus suspension aftera 24 hour period, bioassayed with second-instar larvae.

(B) On Mortality.

The effect on mortality of adding oxalic acid to virus-treated diet surface is shown in Fig. 29.

Although in the preceeding test it was shown that granulosis virus was inactivated in the prescence of oxalic acid, it increased the mortality of larvae due to virus when fed with the virus.

Maximum mortality of virus-infected larvae was observed at 6 mg of oxalic acid per tube, which corresponds to pH 5.2 of the diet surface. A decrease in mortality was observed at higher concentrations.

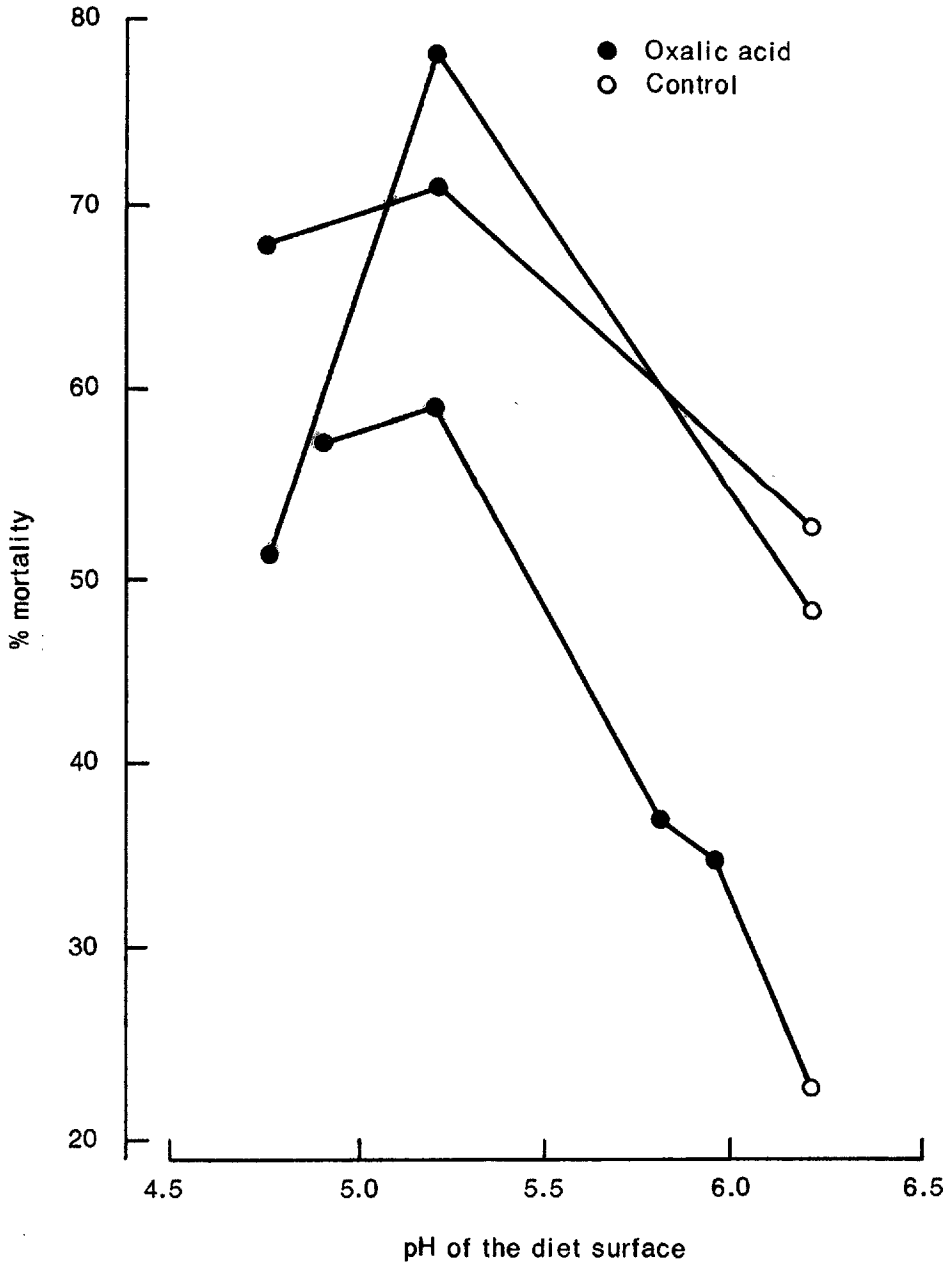


Fig. 29

Mortality of 2nd instar virus-free larvae dosed with granulosis virus on oxalic acid treated diet.

The assay was repeated three times with the results shown in Fig. 29. A total of 1768 larvae were used in these observations.

(c) Effect of Trypsin Inactivators.

The virus and trypsin inactivators were fed simultaneously to second-instar larvae on the surface of formalin-free diet. The proteolytic inactivators did not enhance granulosis virus mortality. (Table 20) Trasylol-S without virus was not toxic to the larvae (Table 20, Control Trasylol 10). The different levels

Protease inhibitor added per tube	No. of larvae treated	Mortality	
		Unidentified (%)	Virus (%)
Trypsin inhibitor soybean, mg			
0.01	198	2.0	39.3
0.1	200	1.0	46.0
1	198	3.5	43.4
Control virus only	198	3.0	45.4
Trasylol-S, µl			
0.05	200	2.5	34.0
0.1	199	3.0	27.6
1	199	3.5	21.1
10	197	3.0	24.8
Control Trasylol 10	200	1.5	0
Control virus only	200	2.0	29.5

Table 20

Effect of protease inhibitors on mortality of second-instar virus-free larvae dosed with granulosis virus.

of mortality reported in Table 20 were probably due to slight differences in age of the second-instar larvae used in the two tests. In both tests the same concentration of virus 3.2×10^5 capsules per tube, was used.

3. Histological and Physiological Observations on the Larvae.

(i) pH Measurement in the Alimentary Canal.

The pH values of regurgitated fluid and of each gut section (Fig. 2) of the virus-free larvae and of three other stocks were separately analysed by the multivariate analysis of variance. Overall significant differences were found at the 1% level for each of the four groups.

The pH values of mid-gut section four (Ab4), because it was higher than others, was compared in each group by one way analysis of variance and least significant differences (LSD), to establish whether significant differences occurred in each individual age group or stock.

(a) pH in the Fourth-Instar Larvae.

The pH of the mid-gut sections of fourth-instar virus-free larvae at 24 hour intervals are shown in Fig. 30.

The small volume regurgitated by the frozen larva was below the minimum required for measurements with the Probion electrodes and its pH was, therefore, not determined. The volume contained in section Th_2 was also too small and its content was divided between Th_1 and Th_3.

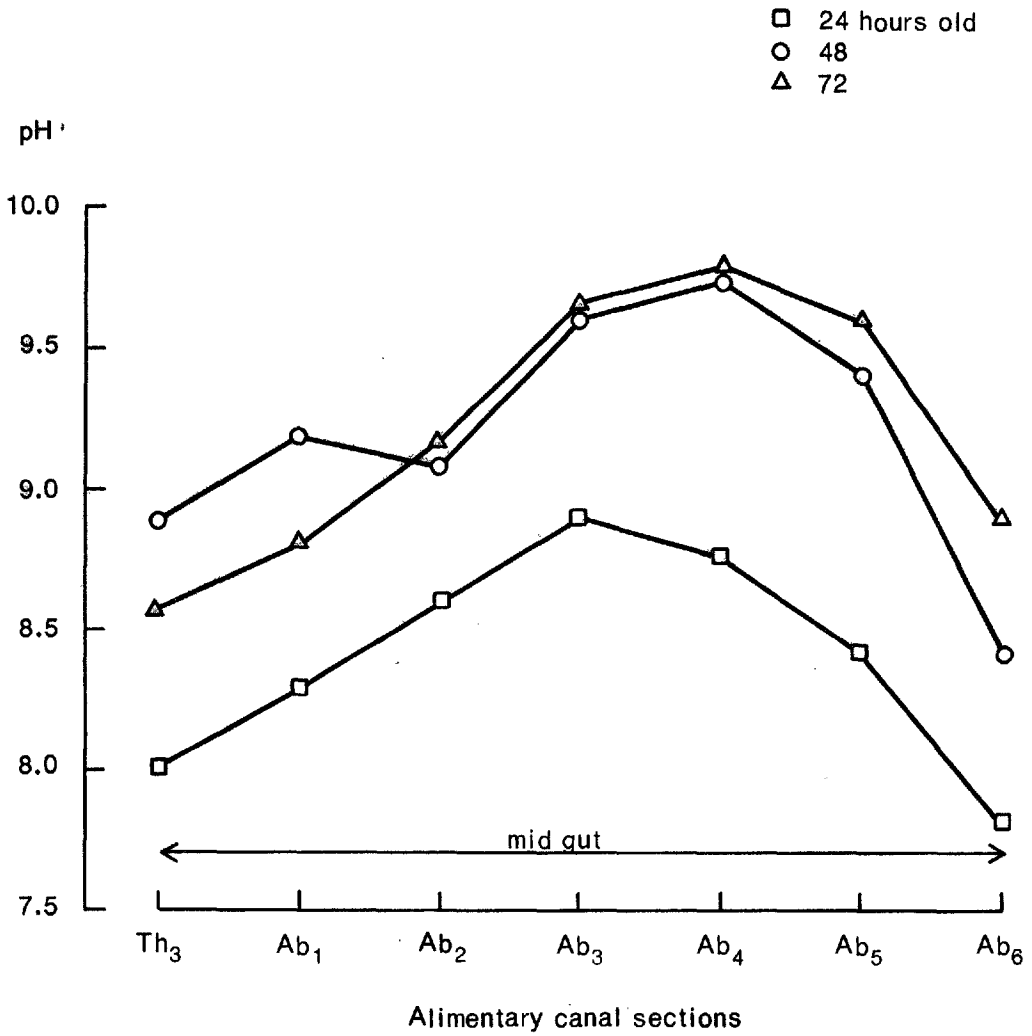


Fig. 30

pH of the alimentary canal of the virus-free stock *P. brassicae*; 4th instar larvae at three different ages.

The average pH at the entrance of the mid-gut (section Th_1) of the 24 hours old fourth-instar larvae was 8, it rose to 8.9 in Ab_3 and returned to 7.8 at the end (Ab_6). The 48 and 72 hours old larvae had similar pH curves but at higher pH levels than the 24 hour old larvae.

Comparing the pH of the Ab_4 sections of the 24, 48 and 72 hours old larvae gave an F-value of 14.88 based on 2 and 16 degrees of freedom which is significant at the 1% level.

The treatment means given in Table 21 show a significant difference between the 24 hours old and the 48 or 72 hours old larvae.

(b) <u>pH of the Regurgitated Fluid, Crop and Mid-Gut Sections of the Fifth-Instar Larvae.</u>

The pH readings obtained with fifth-instar virus-free larvae at three different ages are shown in Fig. 31. Only three curves were chosen to simplify the graph and all the averages of the pH measurements and their standard deviation are given on Table 28 of the Addendum. An overall increase in pH with age was observed and comparison of the Ab_4 section gave an F-value of 11.41 based on 5 and 39 degrees of freedom which was significant at the 1% level. Most of the increase took place up to 96 hours after moulting to fifth-instar, thereafter a slight decrease of pH in the 120 hours old larvae occurred, which in Ab_4 was not significant in the 72, 96 and 120 hours old larvae as

(A) Virus-free larvae			
4th instar		5th instar	
age hours (after moulting)	Average pH	age hours (after moulting)	Average pH
24	8.76 a	4	9.75 a
48	9.76 b	24	9.86 a
72	9.79 b	48	10.10 b
		72	10.25 b c
		96	10.43 c
		120	10.31 c

(B)	
Stock	Average pH
cheiranthi	9.79 a
French	9.80 a
Dutch	10.05 b
Virus-free	10.25 c

Values followed by a different letter are significantly different (LSD at P. 0.05)

Table 21

Comparison of (A) the mid-gut pH of section Ab_4 of fourth- and fifth-instar virus-free of different ages and (B) the four stocks of P. brassicae

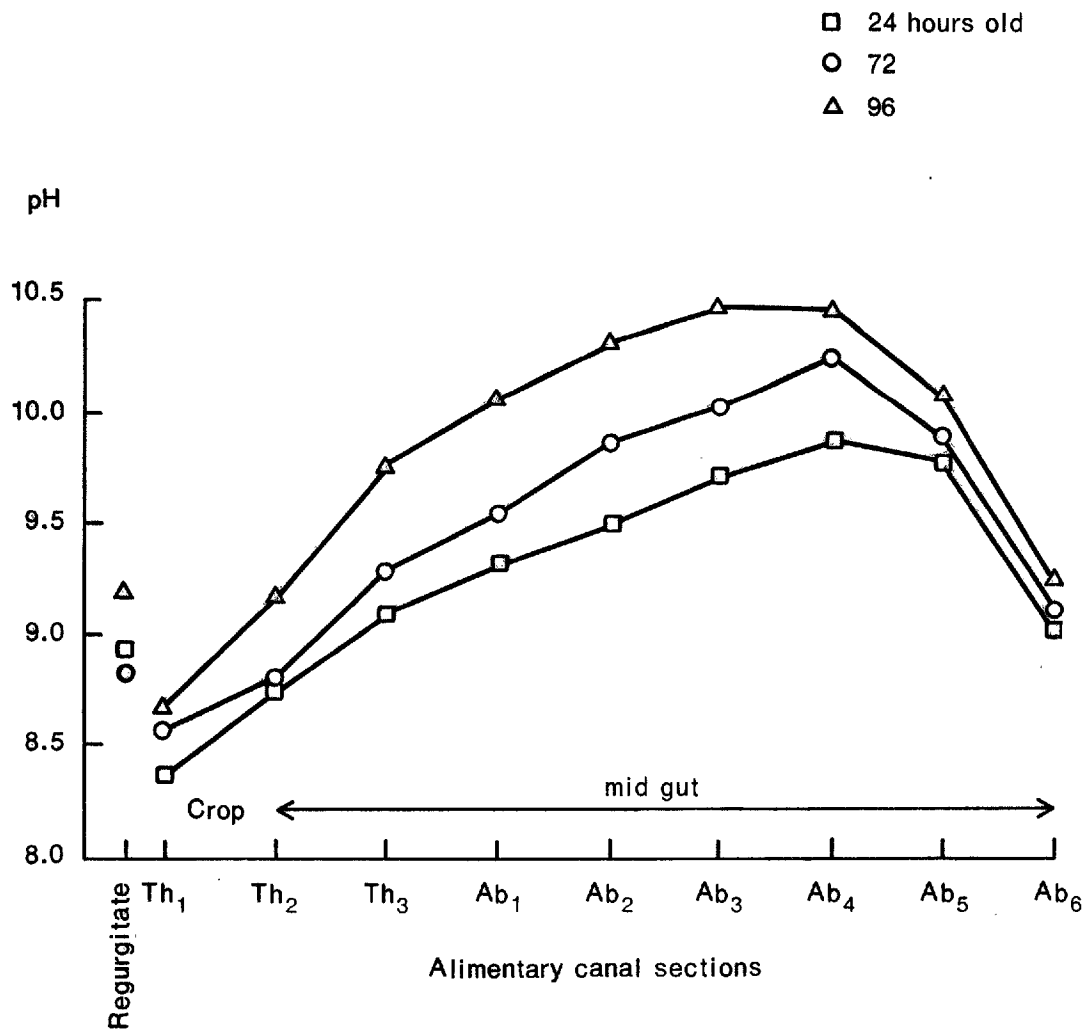


Fig. 31

pH of the alimentary canal of the virus-free stock of *P. brassicae*; 5th instar larvae at three different ages.

shown in Table 21.

The standard deviation values for the average pH in P. brassicae virus-free larvae generally decreased from the regurgitated fluid through the alimentary canal up to the first part of the mid-gut (about Th_3, Ab_1) as shown in Tables 27 and 28 of the Addendum. This is also true for the cheiranthi, French and Dutch stocks (Table 29 Addendum). The highest pH recorded was 10.7 from Ab_4 in two unstarved virus-free larvae 96 hours old.

In separate observations gut fluid was collected individually from 20 fifth-instar virus-free larvae at 24 hour intervals. Before each collection was made the larvae were starved for 1 hour. It was found that the average pH increased with age, ie. pH 10.13, 10.32, 10.47 and 10.52 from 24, 48, 72 and 120 hours old larvae respectively. Also, in these observations, the highest individual pH measured was 10.7 during the last two periods.

The pHs of the crops and mid-gut sections of the various stocks are shown in Fig. 32. Fifth-instar cheiranthi larvae 76 hours old showed the lowest pH throughout, followed by the French, Dutch and virus-free stocks in increasing order.

Using one way analysis of variance, a preliminary comparison of the Ab_4 sections of the four stocks gave a F-value of 12.39 based on 3 and 81 degrees of freedom which

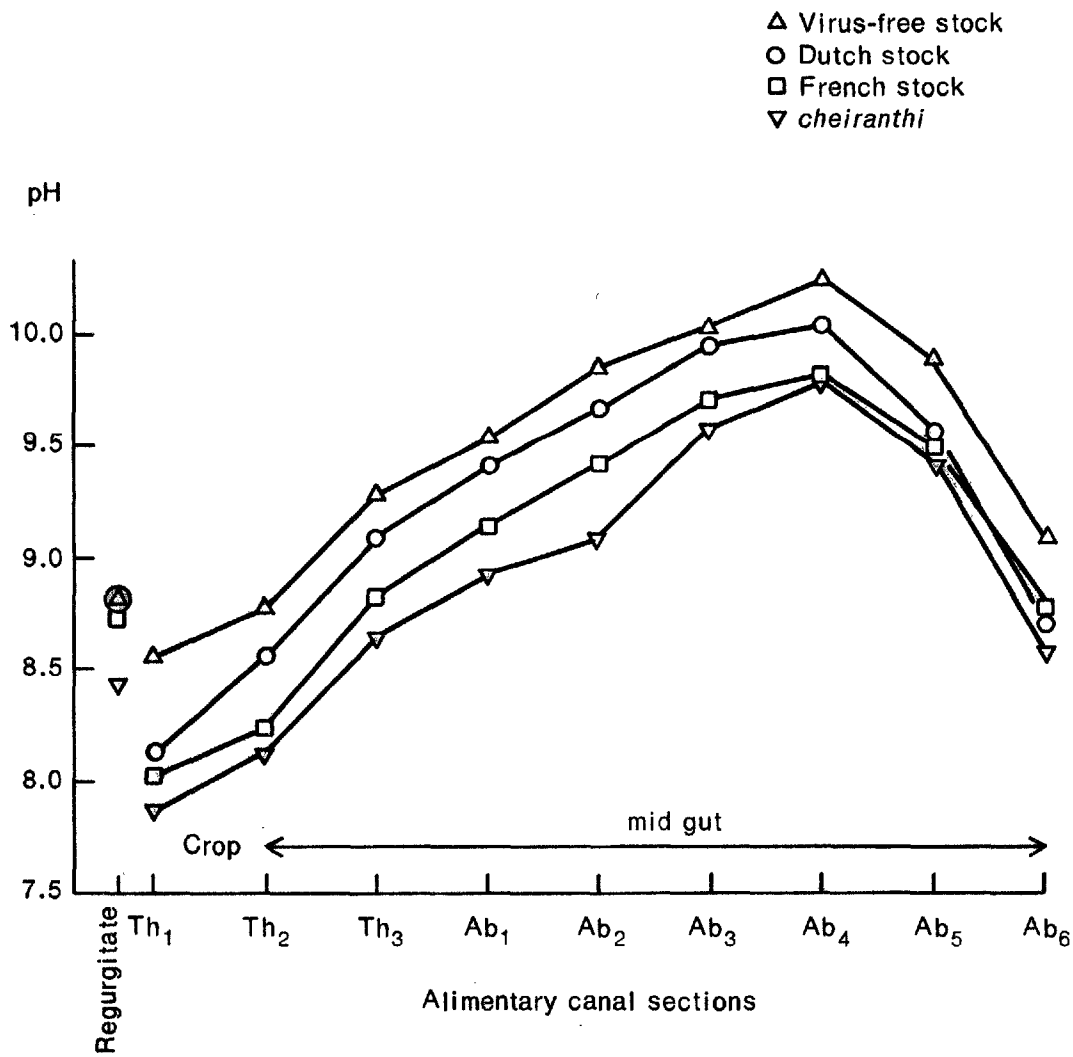


Fig. 32

pH of the alimentary canal of 72 hours old 5th instar larvae of four stocks of *P. brassicae*.

is significant at the 1% level. Employing the least significant difference analysis the Ab_4 section from the cheiranthi stock did not show significant differences from the French stock however the virus-free, Dutch and French stocks were significantly different from each other (Table 21). For sections Th_1 to Ab_3 the pH curve for cheiranthi was lower when compared to the same sections of the French larvae (Fig. 32).

(ii) The Peritrophic Membrane.

Larvae that were about to hatch from the egg were dissected and no peritrophic membrane was detected at this stage. In most larvae the first piece of peritrophic membrane was found surrounding the remaining pieces of skeletonised egg shell ribs while they were still chewing their way out of the egg.

Observations on sections of healthy mid-gut tissue of fifth-instar larvae showed that the peritrophic membrane originates from a group of cells at the anterior end of the mid-gut (Fig. 33). These cells formed a ring at the entrance of the mid-gut and were distinguished under phase contrast because of the reduced number of goblet cells between them and by the small bright areas in the cytoplasm (Fig. 34). This first section of the peritrophic membrane appeared to be diffuse and lacking in uniformity but later as it slid backwards it was more dense and homogeneous (Fig. 35). In almost all the preparations an accumulation

Fig. 33 - 38

P. brassicae mid-gut epithelium

Fig. 33

Origin of the peritrophic membrane (see arrow) (x 300).

Fig. 34

Enlargement of the same area, note small bright areas in the cytoplasm (x 600).

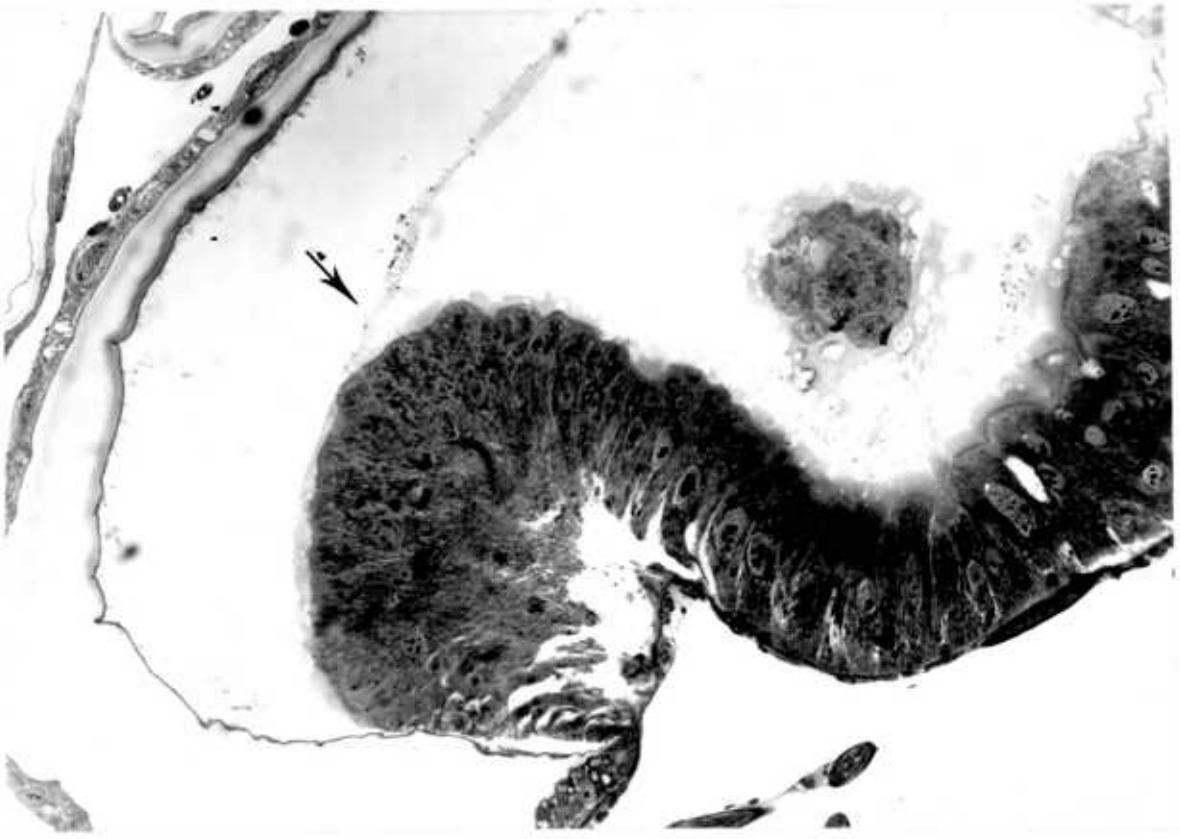


Fig. 33

Fig. 34

Fig. 35

Dense peritrophic membrane a few cells after its initial formation (arrow) (300).

Fig. 36

Accumulation of small bubbles between the peritrophic membrane and brush border. Stained with fuchsin (x300).

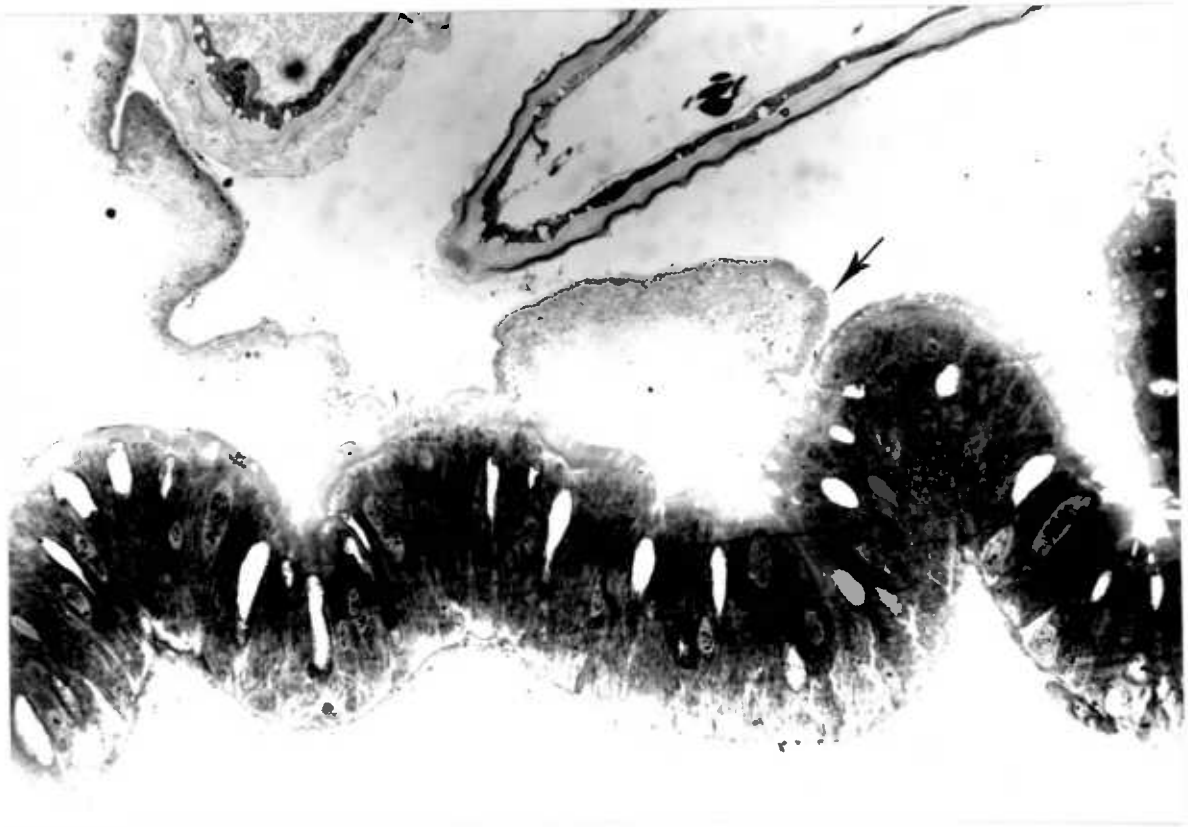


Fig. 35

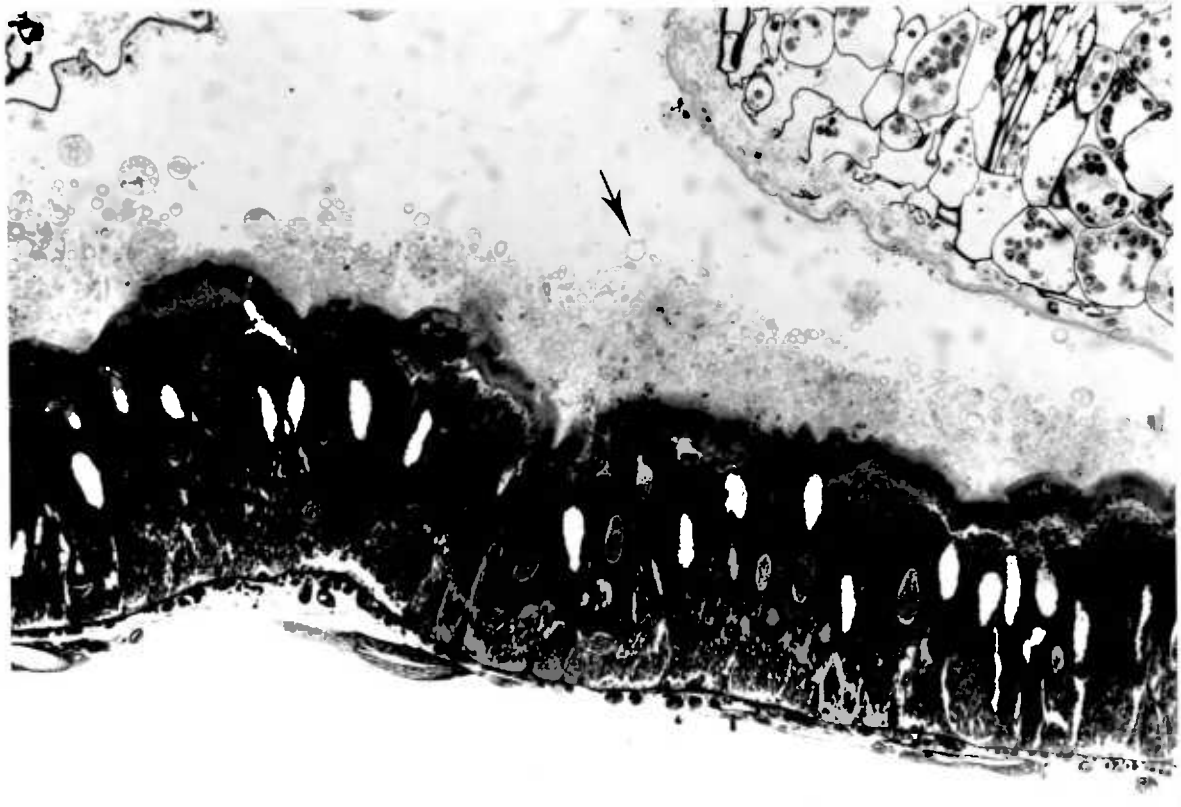


Fig. 36

Fig. 37

Delamination of new sheets from the brush border (x300).

Fig. 38

Active droplike secretion from columnar cells into mid-gut lumen (x600).

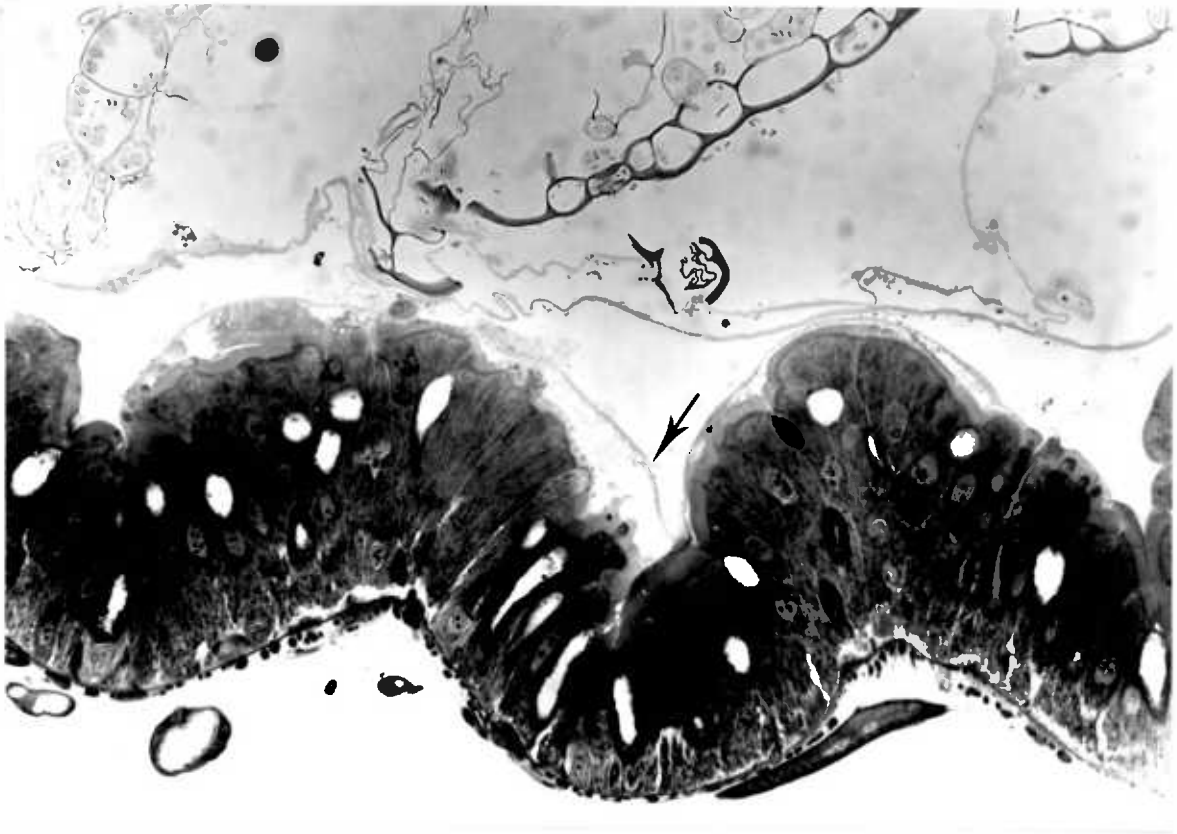


Fig. 37

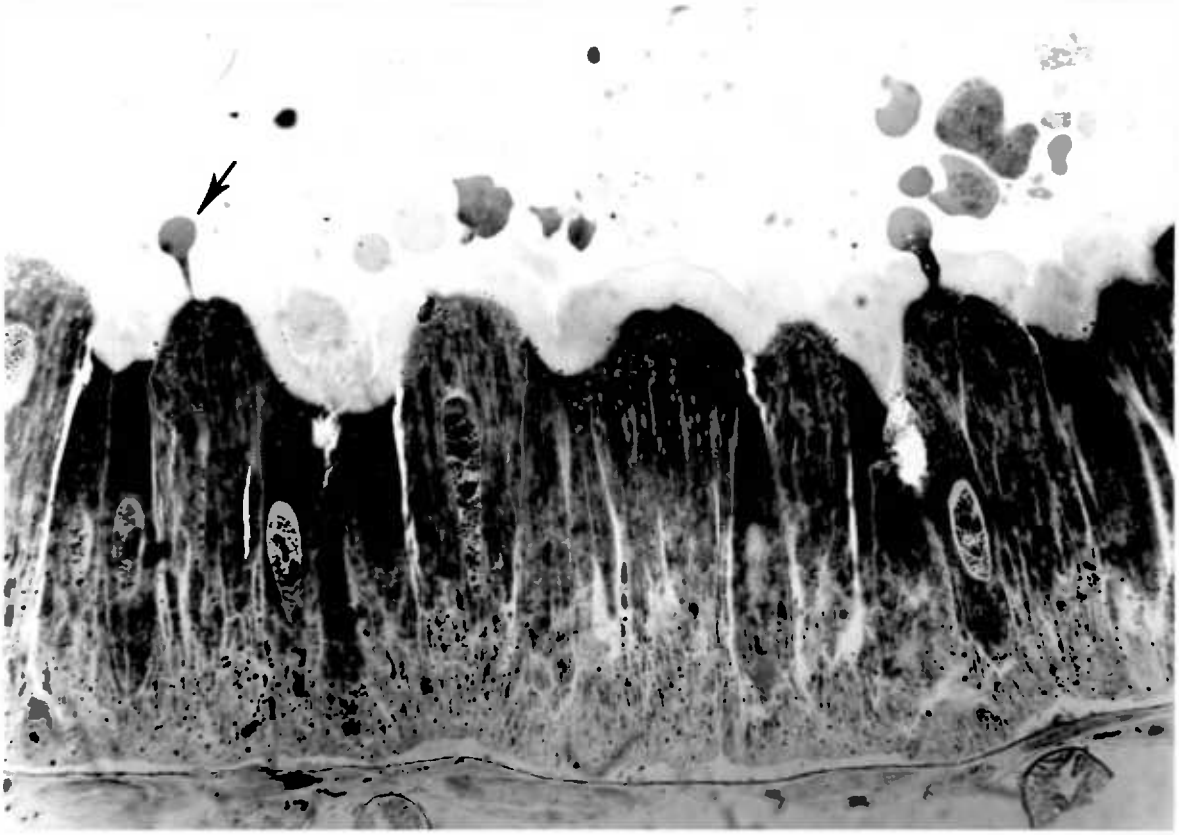


Fig. 38

of material similar to small bubbles was associated with it, generally between the brush border and the peritrophic membrane. (Fig. 36).

After the initial membrane was formed, further layers were added from the brush border (Fig. 37). Some sections of these were not of the same length as the original membrane which was then seen as loops convoluting into the gut contents. Pockets between layers were common but were not found to contain food particles. In the middle part of the mid-gut the peritrophic membrane consisted of several layers probably delaminated successively from the brush border.

No holes, cracks or imperfections were found in sections of the peritrophic membrane observed under phase contrast microscope. Such holes are of interest since they would facilitate passage of virions to the microvilli. Neither were food particles ever observed outside the peritrophic membrane.

(iii) Surface - Volume Relationship of the Mid-Gut.

It was assumed that infection of the columnar cells is most likely to take place in the first half of the mid-gut. The higher pHs in the later sections would inactivate the virus more rapidly making infection here less probable.

The volume and inner surface area of the first half of the mid-gut was calculated from measurements of its diameter and length.

Instar	Volume µl	Inner Surface mm^2	Surface available per 1 µl of gut contents mm^2
late 1st	0.0494	0.642	13.1
early 2nd	0.117	1.212	10.3
late 2nd	0.199	1.919	9.64
late 3rd	0.828	4.914	5.93
early 4th	1.900	8.42	4.43
late 4th	5.97	17.85	2.98
early 5th	10.10	31.27	3.09
late 5th	46.97	77.52	1.65

Table 22

Surface area and volume of the first half of the mid-gut of the five larval instars of P. brassicae.

The proportion between volume and available surface per µl of gut contents is given in Table 22 for each instar. As expected the surface exposed per µl in the lower instars decreased with growth. This difference was almost 8 fold between first- and fifth instar larvae.

(iv) Rate of Faeces Production in the Fifth-instar Larvae.

A newly moulted fifth-instar virus-free larva was placed in each of six 50 ml polycarbonate centrifuge tubes with a hole in the bottom forming a funnel. The inside

surface of the tubes had previously been lined with cabbage leaves supported by a cork. These leaves were changed daily.

The tubes were held vertically in a row of clamps and the falling faeces further directed, by two sheets of non-stick paper, obtained from rolls of sticky labels. They then fell on to bands of sellotape (2.5 cm wide) mounted sticky side up on 50.5 x 26 cm sheets of corrugated cardboard. One of these boards was pulled below the tubes each 24 hours, at a speed of 2 cm per hour, by a string and pulley attached to a time-switch motor revolving once per 24 hours (Fig. 39).

Once the faeces had dried, a cardboard of the same size was placed over the sellotape, trapping the faeces between the sellotape and the second cardboard. The initial cardboard was then detached and the number of faecal pellets produced by each larva every 24 hours was counted, and the figures averaged (Table 23). During the first 24 hours the number of pellets produced was lower, partly because the newly moulted larvae had empty alimentary canals and it took about 2 to 3 hours to fill these and initiate defaecation. Taking this into account the rate at which the faecal pellets were produced changed only slightly as the fifth-instar larvae developed.

After 120 to 145 hours the larvae gradually reduced feeding over a period of 6 hours and became prepupae.

Age of the 5th-instar larvae	Average No. of faecal pellets produced per larva in 24 hours
24	92.6
48	124.7
72	139.2
96	137.7
120	109.8

Table 23

Production of faeces during consecutive periods of 24 hours by fifth-instar of P. brassicae

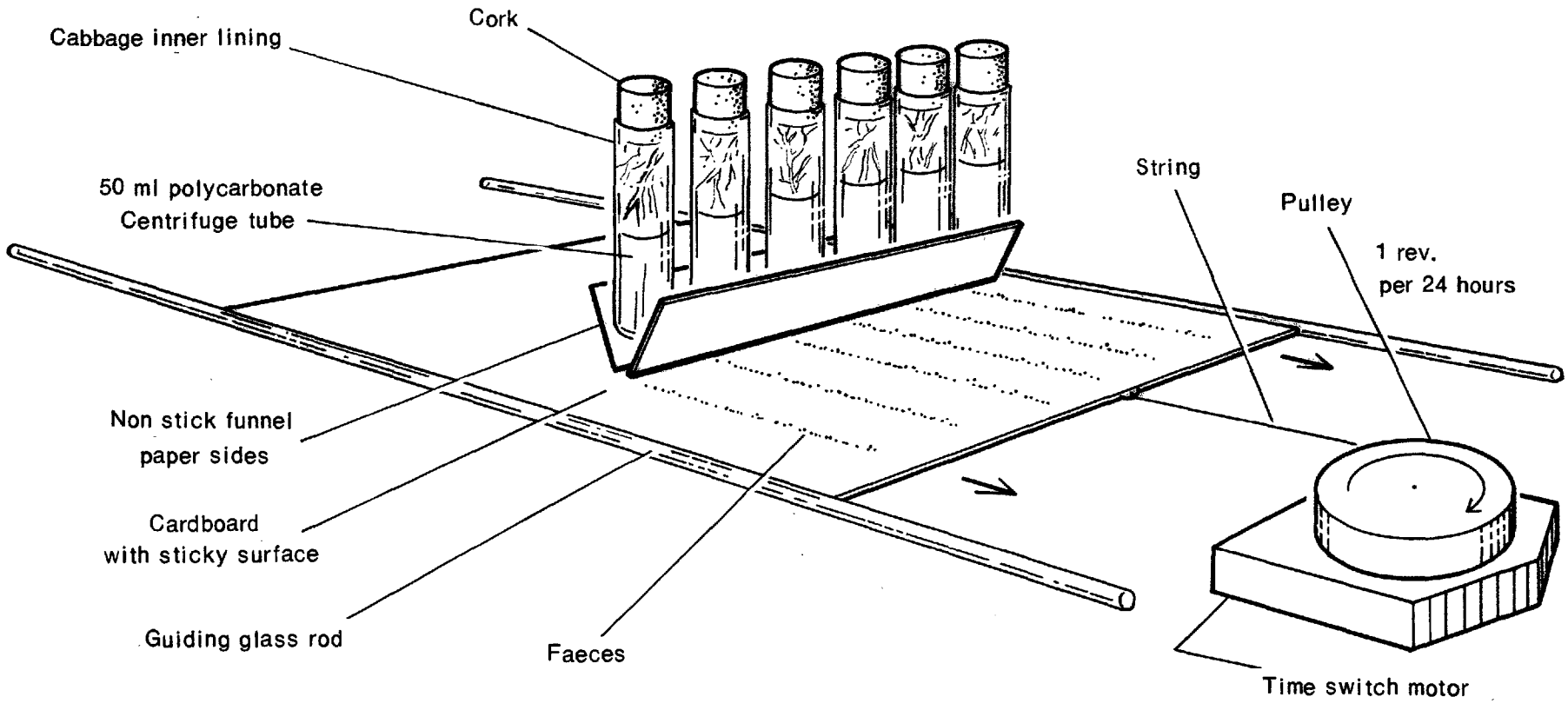


Fig. 39

Apparatus used to record the number of faecal pellets produced by fifth-instar virus-free larvae of *P. brassicae*.

DISCUSSION

1. The Phenol Red Method.

In the studies with P. brassicae it was found that edicol supra black, toluidine blue, sudan III and the two nigrosin stains, were not recovered from the faeces but, phenol red showed promising characteristics and was further tested. Small amounts of phenol red fed to the larvae could be recovered from the frass and strongly stained the extracting solution. A high proportion of the phenol red originally applied to the treated leaf could be recovered from the faeces and from the uneaten piece of leaf.

Crowding 10 to 20 larvae on the cabbage disks reduced the consumption per larva. Thus with 10 and 20 larvae per disk the consumption was 0.0004mg and 0.00035mg phenol red respectively. This is in accordance with the general crowding effect on consumption. Pedigo et al., (1977) indicate that when Plathypena scabra (F.) larvae was kept at either 2 or 3 per container they consumed respectively 12 and 28% less than when reared singly. Although P. brassicae is gregarious in the first 4 larval instars, the crowding level imposed did seem to have an effect on consumption. There appears to be no other explanation for the 4% difference in phenol red recovery at the two larval densities. It is unlikely that the more crowded and probably, therefore, more active group of 20 larvae rubbed off part of the phenol red from the disk surface.

Only 95% of the phenol red applied could be recovered from the dried leaf disks. Possibly, the rest was retained at the rim of the disk in the xylem and phloem vessels which were slightly reddish at the ends after application. The total amount recovered in the calibration control was 0.021mg (Results Table 4) instead of the expected value of 0.025mg. This inaccuracy might have arisen either beacuse the 5 µl micro pipette used showed variations in the delivery or because the concentration of the phenol red solution was slightly low. However, as this control was used as in the calibration, the outcome was not affected.

Using food containing 4% chromic oxide McGinnis and Kasting (1964) indicated that the minimum amount of excreta or food which could be measured by this method was about 5mg of either. When, 0.05% phenol red is added to the diet, less than 0.5mg of food (or faeces) can be measured using 1 ml Spectrophotometer microcuvettes. The phenol red method is therefore 10 times more sensitive, which is especially useful for smaller stages or insects.

The interference in the absorption measurements by faecal matter or its products was studied by comparing phenol red solutions with and without varying amounts of added faeces. An almost constant 4% lower transmission value was obtained. Hence no corrections to the consumption formula (Results 1. (ii)(a)) were made, because 5% of phenol red was not recovered from the leaf or larvae (see Results 1.(ii)(b)) and this almost compensated for

the effect of faeces. It was also estimated that 95 to 99% of phenol red was recovered from second-instar larvae which had fed in uncrowded conditions.

As shown by observations on feeding (Results 1.(ii)(c)) phenol red was not toxic to P. brassicae larvae in the concentrations tested. Koyler (1965) reported that in a feeding test of several colouring compounds that phenol red did not adversely affect adult emergence - the only observation reported. The viability of virus stored at 20 ± 1° C for 9 days with this indicator was not affected and in fact an unexpected higher mortality was observed when compared to the control.

During the spreading of phenol red over standard diet it was absorbed into it. As the diet is an agar based gel it is likely that the virus remains on the surface. This upsets the strict proportionality between the index compound and pathogen and excludes its use when applied on diet surface.

Attempts were made to determine the sensitivity of the phenol red method by varying the concentration of the dye in the diet. No clear differences were detected between treatments, probably because the number of replicates was too small. Larvae ate 39.2mg of the diet containing 0.06% phenol red compared to an average of 31.6mg of diet containing three other concentrations (0.04, 0.05, 0.07%) of phenol red. No explanation was found for this higher

consumption of diet containing 0.06% phenol red. Differences of 0.01% in the concentration of phenol red in the diet could be detected in the frass suspension except as already said, in the 0.06% concentration.

During each larval instar the amount of food consumed when the larvae were reared on diet (Results Table 9) was only half than when reared on cabbage (David and Gardiner 1962). This difference may indicate that the semi-synthetic diet has a higher nutritive value than cabbage.

During the first 24 hours of an instar, consumption by individual larvae varied considerably. The variation was slightly less when measured over the entire instar. This individual variation is also stressed by Rogers et al., (1966) in relation to differing amounts of pathogen consumed in a given time.

The food consumed during the fifth-instar represents the most important contribution to storage of energy. In the present investigation it is shown that the weights of the pupae correlate with the amounts of diet consumed by each larva as determined by the phenol red method - a further proof of the validity of the technique. When working with fifth-instar larvae a lower concentration (0.01 - 0.02%) of phenol red should be used in the diet to avoid having to suspend the excreta in a very large volume of water when measuring its absorption. Conversely with first-instar larvae which produce a small

amount of faeces a higher concentration of dye might be tried.

It is interesting to note that although the virus-free stock of P. brassicae seem very homogeneous after more than 100 generations of laboratory rearing, the larvae still show a remarkable difference in appetite. Although no tests were done with wild P. brassicae, the individual variation in consumption would be expected to be even greater. Considering each larva separately, the amount of pathogens ingested is related to individual appetite which may be governed by, among others, genetic factors. However, even when the dose of pathogen is kept equal for all individuals mortality will vary according to the susceptibility of the individual. Both factors are very important in the survival of the individual but variation in general allows a better chance of survival for the species. For example, larvae that eat less may survive a density of pathogens which would have killed them had they eaten more. But those larvae eating more, in the absence of pathogens are likely to yield adults that lay more eggs, so that the variation within the population provides the insect with means of overcoming different situations. The susceptibility to pathogens among the individuals of a population varies considerably as shown by the slope of the dose-mortality response which is several logs in range. This factor seems to account for the major part of the survival capability of the

species in the presence of pathogens (Fig. 40)

The consumption of diet by P. brassicae larvae containing no added sucrose was slightly higher than the consumption of two diets containing added sucrose as determined by the phenol red method. Thorsteinson (1960) reviewed

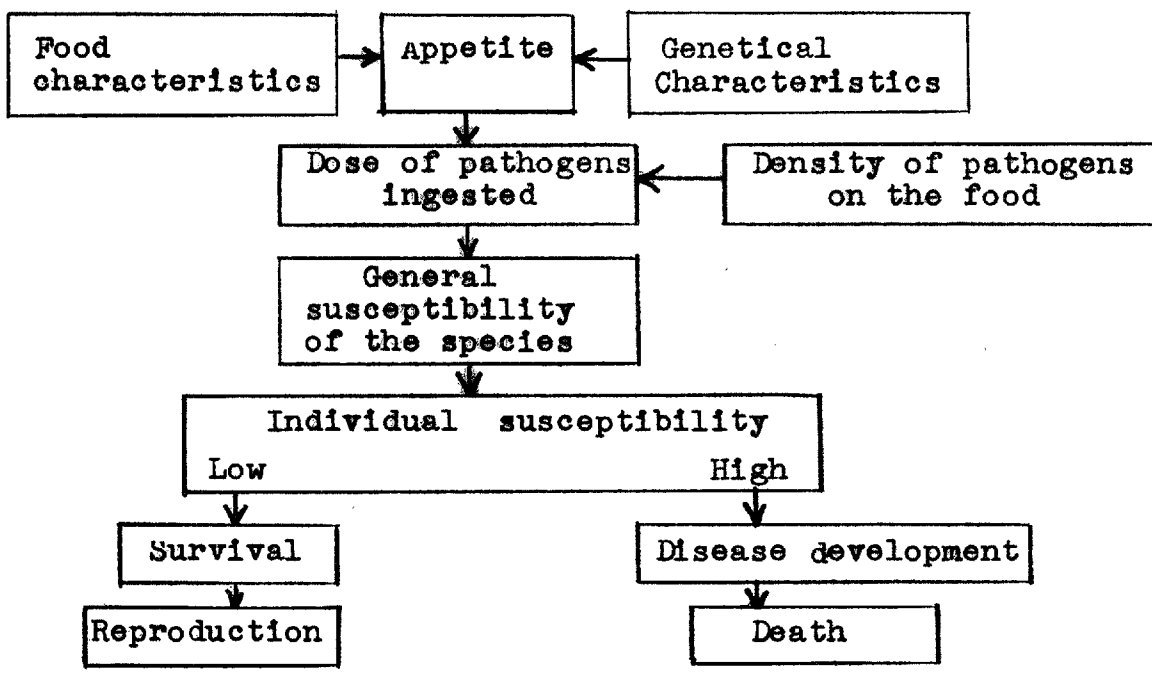


Fig. 40

Abbreviated scheme of the influence of appetite and susceptibility on survival.

the response of several insects to sugars and no general pattern emerged. For example, Plutella larvae manifest a neglibible response to sucrose alone, but sinigrin plus 0.2 M sucrose evoked an appreciable response. Ito (1960) indicates that B. mori larvae feed on artificial diet

devoid of mulberry leaves provided sucrose is added.

P. brassicae larvae feeding on a sugar deficient diet might consume more in order to replace the sucrose with other carbohydrates present in the diet, or it could be that above a certain concentration of sucrose an inhibitory effect occurs (Thorsteinson 1960). Although the larvae feeding on sucrose-deficient diet consumed about 17% more, this difference was not sufficient to explain the increase in mortality of about 21% observed by David et al., (1972a) when comparing the response to granulosis virus fed on normal or sucrose-deficient diets. To achieve this difference (from 42 to 63% mortality) the dose of virus would have to be increased about 10 fold (1 log) (see Fig. 42 in Addendum). A similar increase in mortality was obtained in the present investigation by feeding the virus to the second-inster larvae on acidified diets to lower the mid-gut alkalinity. It is possible that larvae feeding on a sucrose-deficient diet also have a low mid-gut pH which would explain the increase in mortality. The effect of pH on mortality will be discussed later. In concluding this section it can be said that the phenol red method is comparatively simple to use, considerably less time consuming than the chromic oxide technique, cheap and that the only apparatus needed is a spectrophotometer. It seems probable that the method should work with other insects although it has only been used with P. brassicae.

2. Dosage-Mortality Tests.

The dosage-mortality response of second-instar larvae determined using the phenol red technique on leaf strips (Results 2. (i)) gave a close fit to the calculated line. The χ^2 value for heterogeneity was lower than all the independent bioassays with third- and fourth-instar larvae, which were carried out with more than four times as many larvae using the leaf-disk technique.

Assays with second-instar larvae dosed with different concentrations of virus applied to the diet surface following the method described by David et al., (1971b) are consistent with the results obtained in the present study (see Fig. 43 in Addendum). The calculated median lethal concentration (LC_{50}) was 1123 capsules per mm^2 with a slope of b = 0.95 compared to a median lethal dose (LD_{50}) of 1140 capsules/larvae with a slope of b = 1.14 on cabbage leaf with the phenol red method.

The phenol red method of determining the LD_{50} is considerably more time consuming than the leaf-disk method if equal number of larvae are used for both. This is principally due to the colorimetric measurements and because it takes longer to set up the experiment.

Analysis of the dosage-mortality data was based on four groups. This number was adopted after a trial in which more than four groups were used. This failed to give a coherent fit because of the small number of larvae

allocated to each group. The larvae were arranged in groups according to the numbers of capsules ingested, which were calculated from the quantity of phenol red extracted from the faeces. From the data the area of leaf consumed could also be estimated. If susceptibility to virus and appetite were linked characters ie., those larvae which consume above average are also more resistant to virus the analysis outlined would lose part of its validity. It was therefore assumed that if this relationship occurred it was not significant. The close fit of the data to a straight line suggested that the assumption was valid.

Although the experiment was done only once it seemed promising. One of the advantages of the method is that relatively few larvae and required. It also approximates to field conditions where each larvae has the opportunity of ingesting a different virus dose. In the leaf disk method the larvae are, as a part of the routine, starved before dosing and also immediately afterwards when the food carrier is eaten before fresh food is supplied. Usually, also, a variable proportion of the larvae are discarded because they have not consumed the entire dose. However these larvae are part of the natural population and should be taken into account. Furthermore, it is difficult to use the disk method with very small larvae becuase they are delicate and also the very small leaf disks becomes dehydrated before they can be consumed.

Mortality increased directly with dosage in the three larval instars used for the bioassays in the present investigation. A comparison based on the LD_{50} of granulosis virus showed that second-instar were 2099 times more susceptible than third-instar and 53368 times more susceptible than fourth-instar larvae.

The less steep slope of the dosage-mortality line of the second-instar larvae reflects a wider variation in susceptibility when compared with third- and fourth-instar larvae. The slopes of the lines for the latter two instars was similar but third-instar larvae were 25 times more susceptible to the virus than fourth-instar larvae.

The resistance to GV of the virus-free stock of P. brassicae may be compared with that of P. rapae. Thus, Orlob (1973) reported an LD_{50} of 795 capsules/larva for third-instar P. rapae to its own GV, and Jacques (1974) indicated 1.2×10^3 capsules/larva for the same species and instar. From this data and that given for P. brassicae in Table 12 it appears that the virus-free P. brassicae stock is about 1000 times more resistant than P. rapae to their respective granulosis viruses.

Boucias and Nordin (1977) working with Hyphantria cunea and its GV determined an LD_{50} of 7×10^4, 2.7×10^7 and 1.8×10^8 capsules/larva for second-, fourth- and fifth-instar larvae which compares well with the values obtained in the present study. In contract Sheppard and

and Stairs (1977) indicated that the GV of Laspeyresia pomonella, was very pathogenic with an LD_{50} for first- and fifth-instar of only 5 and 49 capsules per larva respectively.

Initial trials were carried out with fifth-instar virus-free larvae but the very high dosage required to achieve around 50% mortality quickly depleted the virus stock so that the tests were abandoned. Wigley (1976) encountered a similar problem of decrease of susceptibility when treating Operophtera brumata fifth-instar larvae with NPV and did not determine the LD_{50}. Even so, the present assays suggested an LD_{50} between 10^9 to 10^{10} capsules/larva which reflects the even higher resistance to virus of the fifth-instar larvae.

Some of the larvae surviving this very high dose of granulosis virus pupated and others became prepupae but did not develop further during the following seven to eight days. A small proportion of these prepupae died of GV disease nearly 14 days after being dosed when the test was stopped. When the haemolymph of the survivors was examined under phase contrast it was found that all were highly infected with virus. In surviving pupae, on the other hand, virus was only sometimes found.

Whitlock (1977) suggests that the resistance to virus of final instar larvae of Heliothis armigera could be attributed to pupation occurring before the virus kills

the larvae. Allen and Ignoffo (1969) also encountered this resistance to virus in the oldest larvae of Heliothis dosed and suggested that "maturation resistance" develops shortly before or during prepupation.

It seems from these observations that the fifth-instar larva approaching the pupal stage, changes in some way which makes it more resistant to infection and subsequent multiplication of the virus. If multiplication is delayed the disease would be slowed down as seen in the prepupal stage. This mechanism probably enables some infected pupating larvae to survive and to complete the development to adults.

No controls were set up in the bioassays mainly because the virus-free stock does not show overt virus disease, and non-virus deaths were negligible among the virus-dosed larvae and other insects observed at the same time. Such deaths as did occur were mainly due to handling. By eliminating the controls more insects could be handled in each treatment.

The change of slope of the time-mortality lines after 9 and 10 days observed with third- and fourth-instar larvae, (Truncation of the lines Figs. 12 and 13) has also been recorded for several other viruses and their hosts (Magnoler 1974, Magnoler 1975, Boucias and Nordin 1977). It was suggested by Sheppard and Stairs (1977) that this truncation was probably due to the development of resistance to virus among individual larvae. This agrees with the observation

of Odier and Vago (1973) that larvae of Galleria mellonella, injected with inactivated densonucleosis virus, developed a non-specific defence mechanism after several days. Furthermore, the range of the incubation time of GV disease varies from 6 to 10 days in third- and 6 to 9 days in fourth-instar larvae respectively for the dosage used. Thereafter the surviving individuals rarely died, a fact which is reflected by the truncation in the time-mortality lines. Younger larvae needed less time to die than older ones, probably because of the smaller body mass and slightly less developed defence mechanism (Salt 1970). A shorter time was needed for initial deaths to occur as the dose was increased probably because once the "infection threshold" (Tinsley 1975) was reached additional virions, infecting the gut cells of the larvae, shorten the incubation time because infection is initiated at more sites.

Probit analysis of the time-mortality response data was not carried out because each line is composed of a cumulative percentage where each value is dependent on the last one, whereas probit dosage-mortality analysis is designed for a line based on independent dosages and mortalities (White personal communication, 1977).

As previously also shown by David and Gardiner (1960) and (1965b), the cheiranthi larvae were the most susceptible of the P. brassicae stocks to GV. Thus with second-instar cheiranthi larvae they reported a derived average mortality

of 72.3%, (David and Gardiner 1965b) with a dose of approximately 680 capsules/larva compared to 79.4% mortality obtained in the present study by feeding approximately 10^3 capsules/larva. Similarly the virus-free larvae used in this study were only slightly more resistant to the virus than those used by David and Gardiner (1965b). This is interesting since there were 12 years between the two investigations during which both stocks underwent approximately 104 generations without major change in susceptibility.

There was a considerable variation in the percentage of second-instar larvae killed by virus with the different batches of larvae of each of the four stocks. This is probably mostly due to slight variations in the average age of the batches of test larvae since David et al., (1971a) showed that a significant increase in resistance to virus was observed between 4 and 24 hour old second-instar P. brassicae larvae.

The mortality figures for fourth-instar larvae of the cheiranthi, French and Dutch stocks deviated considerably from the calculated dosage-mortality line, as shown by the high χ^2 for heterogeneity (Table 15), when compared to the values obtained in bioassays with the virus-free larvae. Since the bioassay method for all stocks was identical this suggests that the larvae of cheiranthi, French and Dutch stocks were less homogeneous than the virus-free stocks.

When the order of susceptibility to GV of the French and Dutch stocks was compared in the second- and fourth-instar larvae it was not the same. With fourth-instar larvae the highest dosage used (when the kill was over 90%) gave a higher mortality with the Dutch than with the French (Fig. 14). However, with dosages giving around 50% kill it was found that the French larvae were more susceptible than the Dutch larvae. This, latter, result agrees with that found when the comparison was made with second-instar larvae. The one higher mortality obtained with the Dutch larvae had sufficient weight on the analysis to change the LD_{50} value and alter the expected order. It also influenced the slope of the line (1.59), which is much steeper than expected, causing it to intersect the dosage-mortality line of the French stock at about the LD_{40} point (Fig. 14). Thus confirming that errors are least when the levels causing 50% mortality are considered, as indicated by Dulmage (1973).

The lower slopes of the dosage-mortality lines obtained with the cheiranthi and French larvae further confirm the conclusion that these stocks also are more susceptible (Burges 1971).

For the above reason it is suggested that the correct order of susceptibility is the one observed with second-instar larvae ie. cheiranthi < French < Dutch < virus-free.

Variations inthe susceptibility to GV are also shown by Sidor (1959) and Rivers (1959) for different stocks of P. brassicae. Burges (1971) gives a critical review of the variation in resistance in different strains of several insects. More recently Lewis (1975), has reported that the LC_{50} for gypsy moths from several geographically different areas varied from 10^3 to 10^5 polyhedral inclusion bodies per ml assessed with second-instar larvae. All these observations suggest that variation in the susceptibility of an insect to a virus is a common phenomenon in nature.

3. Effects of Different Treatments on Virus Viability.

It is well known that alkalis dissolve the granulin of GV and the polyhedrin of NPV and CPV (Faust and Adams 1966, Kawanishi et al., 1972, Paschke and Summers 1975, Padhi 1976). But studies of degradation and loss of viability of the virion in gut fluid seem to give variable results.

The results obtained in the present investigations using either buffers or gut fluid in vitro showed that with an exposure of 1 minute the inactivating effect increases sharply above pH 9.8 (Fig. 16). There were also indications that at a given pH, fresh gut fluid was more effective in inactivating the virus than the same gut fluid after heat treatment or buffer solution (especially between pHs 9.8 and 10.1 (Results Table 16)). The higher gut fluid pHs used may have been proportionally less well maintained

during the tests mainly due to absorption of CO_2, from the air. The likelihood of this occurring was increased because it was necessary to stir the suspension. In the closed environment of the larval gut it is likely that inactivation is even stronger due to longer periods of exposure and CO_2 exclusion. Carbon dioxide was found to lower considerably the pH of P. brassicae larval gut fluid as previously reported by Duspiva (1936).

In three out of six replicates, where gut fluid over pH 10.1 was used to inactivate the virus, which was subsequently fed to second-instar larvae, 0 to 1% mortality was obtained whereas with buffer treatments at the same pH the lowest mortality was 8.5% and that occurred in only one replicate out of six.

After exposure of 1.6×10^9 capsules to gut fluid pH 10.0, which normally occurs in the mid-gut of fifth-instar larvae only an estimated equivalent 2.5×10^5 capsules remained infectious (based on 9.5% mortality of second-instar larvae Table 16). This loss of almost 4 log in infectivity was calculated from the dosage-mortality response for second-instar larvae shown in Fig. 43 in the Addendum. This suggests that roughly one 10,000th of a dose given to a larvae remains infectious after one minute at pH 10.0.

The slightly higher inactivating effect of the gut fluid compared to buffers could be due to the proteolytic enzymes present in the alimentary canal of the larvae

which digests the virions, as suggested by Paschke and Summers (1975). Aizawa (1962) incubated NPV in Bombyx mori gut fluid and found antiviral or inhibitory properties, which could well have been due to proteolytic enzymes. Heimpel (1969) reports that the proteinases in insect gut juice have a direct bearing on the invasive abilities of virus. Summers (1971) indicates that naked virions of GV of Trichoplusia ni are susceptible to degradation when exposed to trypsin under in vitro conditions. Later, Paschke and Summers (1975) estimated a 50 to 60% reduction in infectivity when NPV and GVs are exposed to mid-gut conditions of Lepidoptera. Aruga and Watanabe (1964) reported that CPV incubated in B. mori gut juice pH 10.0 showed only slight antiviral activity. Later Aruga and Tanada (1971) found that CPV dissolves less easily than NPV in alkaline conditions. This probably suggests that the polyhedrin of the CPV is more resistant to the alkaline environment of the mid-gut of Lepidoptera.

Considering the evidence of these authors and the present study it is suggested that the cortical layer of the inclusion body has a special significance : besides protecting the virus from adverse environmental conditions outside the insect, it may also delay degradation of the virus in the gut only very briefly at higher pHs. The envelope also might protect the nucleocapsid from enzyme digestion up to certain extent, as observed by Gipson and Scott (1975) in enzyme treatment of thin sections of embedded NPV.

Based on the catalogue of viral diseases given by Martignoni and Iwai (1977) and the data presented by David (1975), most of the occluded viruses (GV, NPV, CPV, and entomopox viruses) occur mainly in the Lepidoptera and Hymenoptera, which possess generally alkaline mid-guts, and less frequently if at all in Acarina, Orthoptera, Coleoptera and Diptera which mainly have acid or near neutral mid-guts (House 1974). This suggests further evidence for the role of the inclusion body.

Optical density measurements showed that carbonate buffers started to dissolve the granulin of P. brassicae GV at pH 9.4, while Gudauskas and Canerday (1968) using a phosphate buffer recorded 9.8 as the lowest pH at which the polyhedrin of the NPV of Trichoplusia ni dissolved. In the present study carbonate and borate buffers were used and the carbonate was the more effective. An important difference between them at a comparable pH and normality is that the carbonate buffer has more than 3 times as high a sodium concentration as the borate buffer. This difference might be reflected in the time needed to reach the half dissolution point of the virus as measured by the optical density of the suspension ie., half dissolution in the borate buffer took three times as long as in the carbonate buffer. However, this conclusion was based on only one treatment with each buffer, because borate buffer dissolved the virus at only one of the pHs tested. The alkalinity of the mid-gut is probably also based on carbonates (Faust and Adams

1966) and also Na, K, and Mg ions (House 1974) providing, probably, almost ideal conditions for the dissolution of the virus. Paschke and Summers (1975) have reported that although the pH of the gut lumen of the larvae of the Mosquito Aedes taeniorhynchus is neutral, the inclusion bodies of Baculoviruses fed to the larvae are dissolved. However, Dadd (1975) has shown that three species of mosquito larvae, Culex pipiens, Aedes aegypti and Anopheles stephensi held the contents of the mid-sections of the mid-gut at a pH just exceeding 10. It therefore seems probable that Aedes taeniorhynchus had a similar high pH region and that this explains the dissolution and degradation of virions observed by Paschke and Summers. These authors also report that degradation of mosquito iridescent virus became more intense as it progressed from the antherior to the posterior end of the mid-gut. This observation might be explained by the fact that as shown by Dadd (1975) the pH of the mid-gut of mosquito larvae starts at 7.5, increases shortly after to 10.0, and is maintained at this pH for up to 2/3 of the length after which it decreases.

Liu and Zee (1976) also found that vesicular stomatitis virus, imbibed with a meal by mosquitoes was rapidly inactivated in the mid-gut.

In the case of GV of P. brassicae dissolution in carbonate buffer started at around pH 9.4 and infectivity was lost at pH 9.8 after 1 minute exposure. Further evidence of the inactivation of the virus inside the

alimentary canal was obtained from the measurement of the loss of viability of the virus in three Lepidopterous larvae. A calculated dose equivalent to 1.5×10^{10} capsules was spread on a leaf strip measuring 1.8 x 7 cm, assuming that the complete alimentary canal contained at a time 1/10 of the leaf strip (1.5×10^{9} capsules), each of the 5 sections into which it was cut should contain approximately one fifth of this, ie. 3×10^{8} capsules/ section. If no inactivation had occurred this dose should result in about 100% mortality of the 60 second-instar larvae used in the bioassay of each section.

The average inactivation inside the alimentary canal of P. brassicae followed closely the pH condition of the gut. Inactivation among individual larvae varied greatly principally because the pH also varies according to the alternating cycles of eating and resting (see 5. (1) of this chapter).

Based on the highest average inactivation observed in the central mid-gut section of P. brassicae the equivalent of 3×10^{3} capsules per section remained active (0.8% mortality of second-instar larvae). This corresponds to a 5 log decrease in the number of infective particles based on the theoretical number originally present in each section. With the previous in vitro experiments a 4 log decrease was observed. Here, as would be expected, a higher inactivation occurs in vivo.

CPV is considerably more effective when injected into the haemocoel than when fed to B. mori larvae (Watanabe, 1971). To explain this observation Watanabe suggested that much of the virus ingested did not invade the susceptible mid-gut ephithelial cells. The difference based on the data presented by the author as a graph was almost 10^5 fold, which is almost of the same magnitude of inactivation as occurs in the gut of P. brassicae.

Differences in the volume of fluid in the various sections were observed. For example the crop and ileum-rectum sections contained less than either of the three mid-gut sections. However it was estimated that the magnitude of these variations was approximately 2 to 4 fold which in comparison to the magnitude of the inactivation did not seem to be of importance. In addition the highest inactivation was shown to occur in the mid-gut sections which also had the greatest volume.

Compared with fifth-instar P. brassicae larvae, the average pHs of the gut fluids in Lacanobia oleracea and Spodoptera littoralis were slightly lower and the volumes of food contained in each section were at least double. These differences probably explain the overall lower inactivation observed in these two species. The virus inactivation in the individual sections of the gut of L. oleracea did not follow thepH observed except in the case of the first larva bioassayed.

Dissolution studies of granulosis virus in the gut of P. brassicae larvae using the electron microscope suggested

that, in the alkaline conditions observed, the capsules dissolved in one minute or less. This agrees with the dissolution curves in alkaline buffers as determined by optical density measurements in which the initial density of the virus suspension decreased by 50% after only 30 seconds at pH 9.8 at room temperature. Gut fluid seemed to dissolve the capsules more quickly than buffers of comparable pH and, usually, few or no virions were found in these preparations after 1 to 2 minutes. However, a few virions survived as shown in Fig. 22. This observation adds evidence to the virus viability studies in which it was found that the normal gut fluid had a stronger overall degrading effect than buffers or heat treated gut fluid. It seems that after the inclusion body is dissolved the virion has only a very short period of infectivity in the gut fluid. Thus, although the inclusion body is itself rapidly destroyed, it probably survives long enough to afford some protection to the virion except at the highest pHs.

It is difficult to determine the factors responsible for the rapid digestion of the virions in the gut fluid. However, the proteolytic enzymes present in the alimentary canal of P. brassicae (Lecadet and Dedonder 1966) may play some rôle although most GV particles lose their infectivity when subjected only to alkaline buffers as shown in Results section 2 (iii)(a). When these treated particles were observed in the electron microscope the number present was significantly less than expected showing that alkalinity

alone is sufficient to degrade an important proportion of the virions.

The period during which most virions in the mid-gut environment remain infective seems to be very short but, a few survive for longer periods and it is these which have a better chance of infecting the mid-gut cells and initiating the disease. In contrast to these observations, Summers (1971) found that GV virions appeared to be stable in the mid-gut of Trichoplusia ni. However, the pH of the alimentary canal of this species is not known and the significance of the observation is uncertain.

The observations made in this study of the morphological changes of the capsules subjected to alkaline conditions gave similar results to those described by Longworth et al., (1972) for P. brassicae GV

Capsules which are aggregated seem to withstand longer exposures to alkalis. This is possibly due to the smaller area of particle surface exposed in these aggregates. Furthermore, as in the case of polyhedra, the buffering capacity of the granulin (Payne personal communication, 1977) may have a local effect in lowering the pH. It is interesting to compare the observed collective protection afforded by a GV by clumping with that found in NPVs and CPVs where several virions are embedded in one large inclusion body, which probably also provides a collective protection. Besides, it is known that the use of clumped virus prepar-

ations in bioassays may give very misleading results (Vial 1975) which could be due to the varying release rate of virus from clumped and dispersed inclusion bodies.

The virus which had been heat treated in suspension was considerably more resistant to dissolution in alkaline conditions than untreated virus, possibly because of the denaturation of the inclusion body protein. An alkaline protease initially thought of as partly responsible for the dissolution of the inclusion body was not detected in the GV of P. brassicae by Brown et al., (1977) or Payne (personal communication 1977) so inactivation of the virus by heat cannot explain the difference. The dark area observed surrounding the virion in the heat treated preparation (Figs. 24 and 25) probably corresponds to a space, left after the digestion of the inner layer of the granulin by the alkali and enzymes of the gut fluid, which has subsequently become filled with negative stain.

4. Action of Diet Additives on Mortalities of Larvae Fed Granulosis Virus.

Mortality is increased when virus is fed to second-instar larvae on diet acidified with either citric acid plus disodium hydrogen phosphate or sodium hydrogen sulphate instead of normal diet (pH 6.2)

Cabral (1973), observed considerable difference in the mid-gut pHs (ie., from pH 7.1 to 8.6) of larvae of

Porthetria dispar which were proportional to the pHs of the plants on which they had fed. House (1974), reviewed the effect of different foods on the pH of the alimentary canal of several insect species and found that the pH changed only in some of them according to the type of food consumed. Watanabe (1971) reviewed the chemicals that affect the susceptibility of B. mori to virus and among the chemicals tested, acetic acid (0.1 - 0.5 M) increaded the incidence of CPV when the virus was given orally. Based on these observations it may be suggested that the alkalinity of the mid-gut of P. brassicae second-instar larvae feeding on the acidified diet was lowered. The lower pH may have been sufficient to dissolve the capsules while at the same time allowing more of the virus to survive so increasing the infection rate and mortality in comparison with controls fed on standard diet.

Consumption of the acidified diets measured with the phenol red method showed that larvae ate 1/3 less of the most acid diet (ie., pH 3.9). This would have reduced slightly the dose of granules consumed, which partly explains the lower mortality (63%) observed with the diet (Fig. 28). Consumption of the other diets was normal.

Although the GV is inactivated when held in suspension with oxalic acid, a considerable increase in mortality was observed when both were fed to second-instar larvae. The increase in mortality observed probably would have been even greater had the oxalic acid not partly inactivated the virus.

It was also apparent that there was an optimum concentration for maximum mortality (6mg per standard virus dosing tube) above this a further increase slightly decreased mortality. It is suggested that one, or a combination of the following effects, may be responsible for this enhanced virus mortality : The gut pH lowering capacity mentioned for the acidified diets : formation of insoluble salts with calcium and magnesium affecting further the buffering capacity of the alimentary canal, and the possible inhibition of enzymes partly responsible for the inactivation of the virus.

Simple χ^2 tests applied to all replicates, including the tests with acidified diets, showed significant differences only for the assays of oxalic acid treated diet. This chemical introduced new variables, for example by inactivating varying amounts of virus, either on the treated diet surface, or after consumption by the larvae, or both. The result could be that the differences between replicates reached the level of significance.

Lecadet and Dedonder (1966) tested the action of Soybean Trypsin Inhibitor on partially purified gut fluid enzyme preparations of P. brassicae and obtained a maximum of 75% inhibition of the enzyme with the highest concentration of inhibitor used.

It is difficult to measure the inhibition of the proteolytic enzymes in the alimentary canal of P. brassicae after feeding either Trasylol S or Soybean Trypsin

Inhibitor. Larvae were dosed with a two log range in concentrations, but these produced no apparent differences in virus mortalities compared with the controls. There are several possible explanations for this :

a) even if a partial inhibition occurred it may have been compensated for by the secretory capacity of the larvae

b) on the other hand it is also possible that the inhibitors were decomposed in the mid-gut environment before inactivating the proteases due to the presence of other enzymes, micro-organisms or becuase they were absorbed on other proteins present in the diets or alimentary canal (Gilmour 1961), and

c) that the proteolytic activity is not important for virus inactivation.

No conclusion was reached in the present investigation.

However, methods should be developed to evaluate "in vivo" the inhibitory capacity of these and related substances. If they are found to be active their effect on uptake of virus from the gut could be investigated.

5. Histological and Physiological Observations of the Larvae.

(i) pH of the Alimentary Canal.

From a study of the literature it appears that the use of instant freezing with liquid nitrogen to kill the larvae and preserve the gut contents almost unchanged for pH measurements is an innovation. The advantages of the

technique have already been discussed in Materials and Methods.

The mid-gut pH of P. brassicae as given by Skrjabina (1936) and Staudenmayer and Stellwaag (1940) (see Waterhouse 1949), was 9.4 and 8.0 respectively. Both values are considerably lower than those determined in the P. brassicae virus-free stock using the freezing technique and micro glass electrodes.

The pH of the mid-gut of first-, second- and third-instar larvae was observed following a method similar to that used by Dadd (1975) for mosquito larvae (pH 10.1). Two of the five pH indicators used (ortho-cresolphthalein and thymolphthalein) showed no change in colour. Although the method lacked accuracy it was estimated with the other three indicators (metacresol purple, thymol blue and alizarin yellow R) that the maximum pH in the mid-gut of first- to third-instar larvae was between 9.0 and 9.6. The colour changes suggested that the pH in the third-instar larvae was only slightly higher than in the first- or second-instar larvae. The cycles of pH which occur with feeding and resting (see below) were however easily observed and the changes in pH affected the crop and anterior mid-gut up to section Th_3.

It took considerably longer to measure the pHs of the gut contents of fourth-instar larvae than of the larger fifth-instar larvae and it was necessary to use the cooling

device to prevent the larvae thawing during dissection.

When the curves (pH in the crop against mid-gut sections) for the 48 and 72 hours old fourth-instar larvae were examined (Fig. 30) it was found that these overlapped. This was probably because the 48 hours old larvae were about to resume feeding, and hence had a higher average pH (see below). All the larvae observed showed distinct cycles of pH in the crop and in the first part of the mid-gut, related to the periods of feeding and resting which are characteristic of many Lepidopterous larvae (Chapman 1974). This oscillation is reflected in the standard deviation (S.D.) of pH values for various gut samples of fifth-instar larvae (Table 28 Addendum). These are generally highest for the regurgitated fluid, less in decreasing order, in the crop and in the anterior mid-gut sections (Th_2 and Th_3 approximately) and thereafter stable. These results can perhaps be explained by the fact that the food consumed during each feeding period almost fills the alimentary canal up to Th_3 section.

A larva which is about to resume feeding shows higher pHs in the three anterior gut sections than just after feeding. The values then fall gradually as the larva feeds because of the slight acidity of the cabbage leaves or diet. At the end of the eating period the pH is also lowest in these anterior sections, but it then gradually increases during the resting period. When 4 hour old fifth-instar virus-free larvae were examined they did not show the

decreasing order of the S.D. values previously reported for older larvae (Table 28 Addendum) probably because most of these larvae had not fed after moulting to fifths before the pH measurement. This would also explain the comparatively high pHs found in Th_1 to Ab_1 sections which were similar to those of the remaining mid-gut sections.

Larvae which had been starved for 1 to 3 hours were found to regurgitate a more alkaline fluid than unstarved larvae, probably because, in this extended resting period, the pH of the anterior sections continued to increase in preparation for the next meal. Waterhouse (1949) in his review of the alimentary canal pH of Lepidoptera reports that starved B. mori larvae also had a higher mid-gut pH than unstarved larvae.

The pH of the fluid obtained when larvae were made to vomit by exciting them was considerably higher (pH 10.3 to 10.5) than that of the fluid regurgitated when larvae were plunged into liquid nitrogen. In the former case the fluid was probably also expelled from sections near Ab_4 where the pH is highest.

The distinct increase of pH in the mid-gut observed between fourth- and fifth-instar larvae of P. brassicae was also noted by Heimpel (1955) in Malacosma disstria Hbn, where the maximum pHs were 10.17 and 10.31 for fifth- and sixth-instar larvae, respectively. Lewis and Cannola (1966) reported that Porthetria dispar (L) showed in fourth-, fifth-

and sixth-instar larvae maximum pHs of 7.41, 8.22, and 8.69 respectively. Burton et al., (1977) give the average pH of the mid-gut Heliothis zea larvae of various ages. The values ranged from pH 8.07 for 5 day old larvae to pH 9.27 for the 10 day old larvae which were presumably, in the last instar. On the following day, (one day before the formation of the pupae), the pH had fallen to 9.08. A similar decrease in pH just before pupation (in 120 hour old fifth-instar larvae), has also been observed in P. brassicae. The authors also found that, as in P. brassicae, the average pH of the gut increases from one instar to the next.

Observations show that in successive larval instars the pH of the gut of P. brassicae increases and that the LD_{50} values for granulosis virus also increase. Taken together with in vitro viral inactivation studies, these facts suggest that the pH of the alimentary canal is an important factor limiting the number of virions that actually infect the mid-gut.

If this explanation is valid the increase of pH observed in a particular instar would explain the increase in resistance to the virus over a period of 24 hours observed in second-instar larvae of P. brassicae (David et al., 1971a). Allen and Ignoffo (1969) also found a daily increase in resistance of H. zea to nucleopolyhedrosis virus. Both these observations support the suggestion that the alimentary canal pH influences the resistance of a larva to GV and probably,

also, to NPV.

The average pHs of the guts of fith-instar larvae of the cheiranthi, French, Dutch and virus-free stocks increased in that order as did their comparative resistance to granulosis virus as determined with second-instar and fourth-instar larvae. (The slight difference in order which occurred in the tests with fourth-instar larvae of the Dutch and French stocks, was probably due to an error as explained in section 2. of this chapter.)

Stocks of P. brassicae which differ in susceptibility to GV can be usefully employed to study the factors which govern resistance. Stocks which have low resistance can be used with advantage in the mass production of virus since less inoculum will be required. For example, if fourth-instar cheiranthi larvae were used instead of the virus-free stock at LD_{50}, 5.6×10^{10} capsules would be saved for each 1000 larvae dosed. There is also the possibility that in a different stock of larvae different selection pressures would be imposed on the virus and that after several passages the characteristics of the virus would be changed.

Individual variations in the mid-gut pH, probably of genetic origin, were apparent from the data. This is considered as one of the factors contributing to individual variation in resistance to GV. As suggested previously a high pH in the mid-gut reduces the chances of infection

with GV. In these larvae a greater proportion of the virus is degraded and the "infectivity plateau" (Tinsley 1975) may not be reached.

It has to be stressed that pH is not the only factor which determines whether or not infection and ultimately death occurs. The peritrophic membrane and other defence mechanisms such as phagocytes (Salt 1970), also probably play an important rôle. And although alkalinity alone inactivates the virus, proteolytic enzymes in the gut juice whose activity is almost proportional to the pH (Lecadet and Dedonder 1966), probably increase virus degradation, especially at high pH.

If the pH of the mid-gut is an important factor in the defence mechanism of insects to virus then the resistance observed in some stocks of P. brassicae (David and Gardiner 1960 and 1965b, Rivers 1959, and Sidor 1959) may have resulted from the elimination of suspeptible individuals having a low pH. Breeding from the survivors of a stock repeatedly exposed to viruses would ultimately result in a stock with a higher average pH and resistance.

This mechanism probably explains the resistance to virus found in the virus-free stock (David and Gardiner 1966b and 1960) which was initially selected from a contaminated stock. Sidor (1959) found that three stocks of P. brassicae which showed a marked difference in resistance to granulosis virus showed the same order of resistance to a CPV. Watanabe

(1971) found that the order of susceptibilities of several silkworm strains to NPV and CPV were significantly related. He therefore suggested that the mechanisms of resistance to both virus infections were substantially similar and as suggested below may have been partly determined by the pHs in the guts.

To determine whether there is a general relationship between LD_{50} and mid-gut pH, both values are given in Fig. 41 for Lepidopterous larvae, where both are known. Unfortunately not all the pH figures are reliable so that the relationship might be affected. Thus, for P. rapae Grayson (1951) gives a pH of 7.3 to 7.6 in contrast to pH 9.9 found for the center mid-gut in the present study. Also the determinations of dosage-mortality and gut pH for a given species were not always carried out on the same instar. It was therefore necessary to introduce an arbitrary correction factor by which the pH was adjusted by 0.3 units per instar (based on data from Heimpel 1955, Lewis and Connola 1966 and Burton et al., 1977) if both were not given for the same instar. In the case of Porthetria dispar several authors have determined the LD_{50} (Bergold 1953, Doane 1966, Stairs 1972, Magnoler 1974, and Padhi and Chase 1976). However the value given by Magnoler (1974) was adopted because it included the mortalities up to the point where the time mortality regression line levelled off.

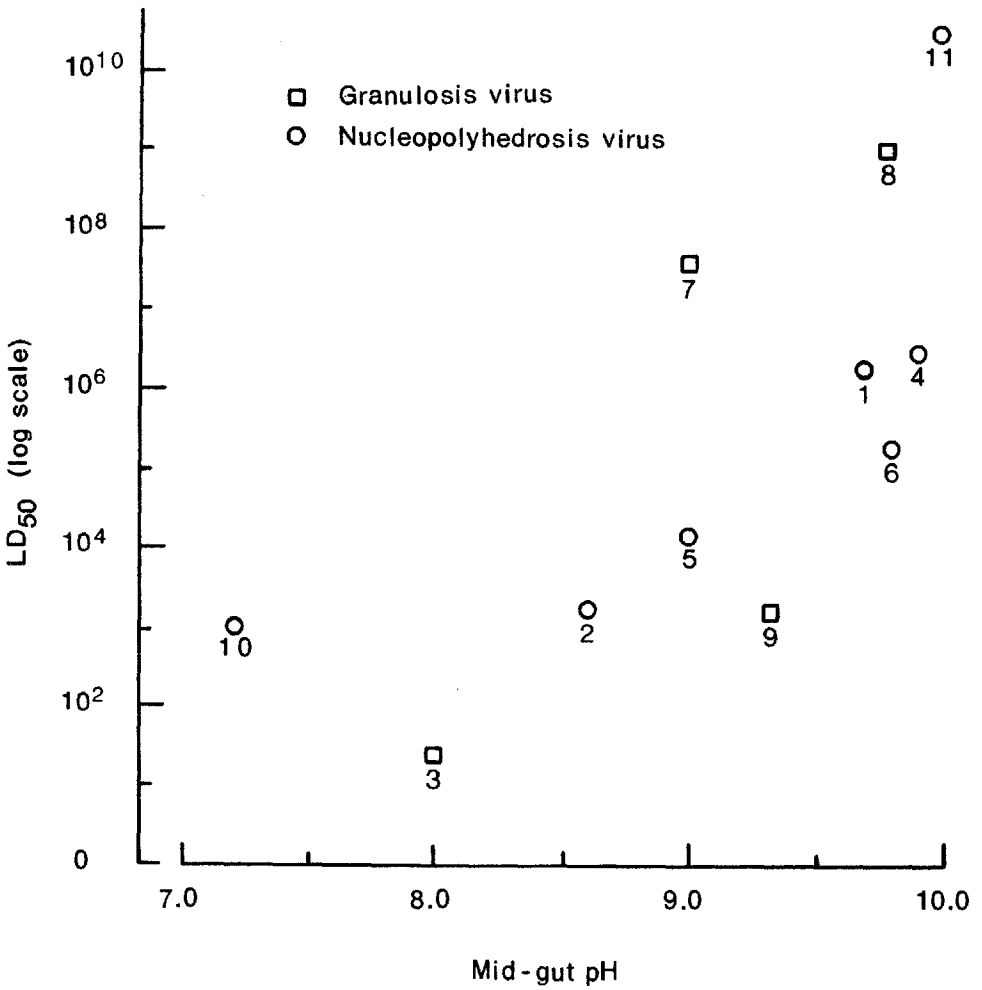


Fig. 41

Relationship between susceptibility to viruses and mid-gut pHs of Lepidopterous larvae (For explanation of numbers see Table 24)

TABLE 24

Sources of the data on the dosage-mortality response, and mid-gut pHs used in Fig. 41. The particular larval instars employed are also given.

Species	Identification No. in Fig. 41	Dosage - Mortality		Mid-gut pH	
		Instar or Stage used	Reference	Instar or Stage used	Reference
Bombyx mori	1	3rd	Morris 1962	5th	Heimpel 1955
Heliothis zea	2	88 mg 8 day	Allen and Ignoffo 1969	98 mg 6 day	Burton et al., 1977
Laspeyresia pomonella	3	5th	Sheppard and Stairs 1977	5th	Heimpel 1955
Malacosoma disstria	4	4th	Stairs 1965	5th	Heimpel 1955
Malacosoma neustria	5	4th	Magnoler 1975	5th	Waterhouse 1949
Mamestra brassicae	6	5th	Gröner 1976	5th	Ripa 1977 unpublished

Continued on next page

TABLE 24 (continued)

Sources of the data on the dosage-mortality response, and mid-gut pHs used in Fig. 41. The particular larval instars employed are also given.

Species	Identification No. in Fig. 41	Dosage - Mortality		Mid-gut pH	
		Instar or Stage used	Reference	Instar or Stage used	Reference
Pieris brassicae	7	4th	present study	4th	present study
Pieris brassicae	8	5th	present study	5th	present study
Pieris rapae	9	3rd	Jacques 1974	5th	Ripa 1977 unpublished
Porthetria dispar	10	3rd	Magnoler 1974	4th	Lewis and Cannola 1966
Spodoptera littoralis	11	6th	Zeya 1971	6th	present study

The correlation between pH and the LD_{50}s ($r = 0.694$ based on the log of the LD_{50}) evident from Fig. 41 further suggests that the degree of alkalinity in the mid-gut has an important rôle in the defence mechanism of insects against virus infection.

(ii) The Peritrophic Membrane.

To study the peritrophic membrane it is necessary to cut sections of the gut. Embedding the mid-gut tissue in methacrylate gave a better preservation than embedding in Paraplast when observed with the light microscope. The improved cutting properties of the tissues in methacrylate permitted longitudinal mid-gut sections 1 micron tick by 10 mm long to be cut. This enabled almost half of the peritrophic membrane present in the alimentary canal of a fifth-instar larvae to be observed. However, only a few staining procedures, outlined by Dawes (1971), for expoxy sections can be used, in contrast to the variety available for paraffin sections. Peritrophic membrane seems to appear in P. brassicae when the first solid food (ie., the egg shell consumed by the hatching larvae) is ingested (Results, section 3. (ii)). In Drosophila secretion of the peritrophic membrane begins in the embryo and in Aedes aegypti it starts in newly hatched larvae (Richards and Richards 1977). No information has been found in the literature about its time of origin in Lepidopterous larvae. Microscopic observation of the contents of the gut at this stage revealed the sharp ribs of the pieces of

shell which might easily damage the mid-gut wall if it was not protected by the peritrophic membrane. Also, when the larva starts eating it begins to be exposed to micro-organisms some of them possibly pathogenic, against which the peritrophic membrane acts as a barrier.

In the course of the present investigations the origin and formation of the peritrophic membrane in P. brassicae has been investigated.

Peters (1969) reported the structure of the membrane when fully formed to be of an irregular felt-like, dispersed texture. The soft and probably still semi-fluid state of the peritrophic membrane of P. brassicae at its origin may allow the virions to penetrate it at this point and so reach the mid-gut cells. A few cells further on, the membrane gains consistency and, as it slides backwards new sheets are delaminated from the brush border, giving it additional thickness.

Even if virions could pass through the several layers of the peritrophic membrane, which exists at the center of the mid-gut, the new sheets that might be delaminated would tend to trap the virus between the layers, if the pores of this last sheet are sufficiently small.

In P. brassicae larvae the pH increases from the entrance until just posterior to the center of the mid-gut. Thus as it passes through the gut to this point the virus is exposed to progressively more inactivation conditions.

This suggests that the barrier is likely to be crossed before the center of the mid-gut. In addition Tanada et al., 1975 reported that 2 to 4 hours after feeding NPV to Pseudaletia unipuncta, the first virions were detected in the anterior mid-gut, between the peritrophic membrane and the mid-gut epithelium. Richards and Richards (1977) state that the digestive enzymes of the gut partly disintegrate the peritrophic membrane. In some cases this may be of considerable importance in assisting the virus to pass through it. Perhaps the continually delaminating sheets observed in P. brassicae act as a reinforcing mechanism for the damage occurring at the same time to the, older, internal layers. It is also interesting to note that the CPV of B. mori generally first infects the posterior cells of the mid-gut (Iwashita 1971). This virus is also more tolerant than GV or NPV to alkalinity (Kawase 1971) suggesting that, in this case the virion might cross the peritrophic membrane after the latter has been partly digested by the gut enzymes.

Richards and Richards (1977) found that the peritrophic membrane of first-instar mosquito larvae is thin and devoid of a fibrous grid, whereas in the later instars the peritrophic membrane is thicker and has fibrous layers. In the present study of P. brassicae it was found that the peritrophic membrane of first-instar larvae was delicate and difficult to remove complete, in contrast to the membrane of fith-instar larvae which was well-defined and easily

removable.

The pore sizes of the peritrophic membranes reviewed by Richards and Richards (1977) seems to lay between 2 and 9 nm in diameter, based on its permeability to different compounds. P. brassicae GV virions are much larger (350 x 50 nm, Brown et al., 1977) which excludes the possibility of them passing through the pores.

Finally it may be concluded that the increase in thickness of the peritrophic membrane which occurs as the larvae age probably accounts for some of the simultaneous increase in resistance to virus infection.

(iii) Surface-Volume Relationship of the Mid-Gut.

The study of the change in surface-volume relationship of the mid-gut as the larvae developed showed that first-instar larvae had 8 times more surface per µl of volume of gut contents than a fifth-instar larva. If the virions in the periphery of the gut lumen alone are likely to infect the microvilli, and these are supposed to occur in a tubular layer 0.01mm thick, this relationship decreases to a difference of only 1.7 times between first- and fifth-instar larvae. For the purpose of this comparison it was assumed that the microvilli exposed a surface that remained equally susceptible to virus infection throughout the larval development. If the surface-volume relationship for second-instar larvae to fifth-instar larvae, is compared with the

difference between the LD_{50}s which is about 6 log, it is apparent that the surface-volume ration has, little or no significance in the increase of resistance which occurs with larval development.

(iv) Rate of Production of Faecal Pellets by Fifth-Instar Larvae.

The apparatus for collecting faecal pellets (Results 3. (iv)) provides a continous record of the production of faeces. The phenol red method, if used continously throughout the fifth-instar would have been more laborious and would have given a less detailed record of the consumption.

An expected increase in the size and number of faecal pellets produced per 24 hours was observed until the fourth day. Since the size as well as the number increased, the rate of food consumption was not adequately shown by the number of pellets produced. Observations of the size of the faecal pellets indicated that the volume roughly doubled between 24 and 96 hours. Hence the amount consumed in one day by fifth-instar larvae which have moulted 96 hours previously would be about three times as much as for larvae which had moulted 24 hours previously, because the number of faecal pellets also increased.

Although there is a small change in the rate of passage of food through the gut with age in the fifth-instar larvae (and probably also in other instars) it is not possible to correlate this change with an alteration in response to virus

on the food. There are several reasons why this is so. The increase in rate itself is comparatively small and probably insufficient to have a detectable effect on mortality. An increased rate of passage of food would mean that the virions reach the high pH zone sooner, and stay in the mid-gut, where infection occurs for a shorter time. Both factors would tend to influence virus uptake adversely.

However further studies are required to determine the effect which the rate of passage of food through the gut may have on virus uptake.

6. Suggestions for Further Investigations.

Arising from this work many suggestions could be made regarding further investigations. However, one subject is of outstanding practical importance.

It seems that only a small fraction of the virus dose infects the larvae especially in later instars. Apparently the loss of infectivity of the virus is almost directly related to the pH of the larval mid-gut.

It has been shown in this sutdy that acidifying the medium on which the virus is fed increased virus mortality. Sub-lethal doses of some insecticide have also been shown to decrease the mid-gut pH (Berim and Bykhovets 1971).

Taken together these observations suggest that insecticides which lower gut pH might enhance the effectiveness of pathogenic viruses. The advantages of combining sub-lethal doses of insecticides with viruses have been reviewed by Benz (1971).

A further, more detailed, study of the advantages of combining sub-lethal doses of insecticides with viruses seems to be fully justified.

SUMMARY

Pieris brassicae was reared in the laboratory, usually on semi-synthetic diet, but some times on the food plant. The following four stocks were used : the virus-free, Dutch, French and *cheiranthi*.

Granulosis virus (GV) was produced by dosing fourth- and fifth-instar larvae with crude virus. The virus was purified by filtration, differential centrifugation and finally on sucrose gradients.

A colorimetric method was devised to measure food consumption by larvae. Phenol red was incorporated into the diet and the amount of dye excreted (and thus, the amount of food consumed) was measured from optical density of an alkaline extract of the faeces.

This colorimetric method was used to measure : average food consumption of second-, third-, fourth- and fifth-instar virus-free *P. brassicae* larvae, the recovery from faeces once applied to leaves ; the toxicity of phenol red to larvae and to the virus ; and the effect of sucrose content in the diet on consumption.

Larval susceptibility to GV was determined using the dosage-mortality response. For second-instar larvae the dose consumed was determined by the colorimetric method mentioned above. For third- and fourth-instar larvae fixed

virus dosages were administered to the larvae. Considerable differences in susceptibility to GV were found between second- and fourth-instar virus-free larvae. Data on the time-mortality responses are given for third- and fourth-instar larvae.

Comparative bioassays with the four different stocks using second- and fourth-instar larvae, showed that the cheiranthi was the most susceptible followed, in decreasing order, by the French, Dutch and virus-free stocks.

The pHs of the nine sections of the alimentary canal of fourth- and fifth-instar larvae were measured. The larvae were frozen in liquid nitrogen, cut into sections and the gut content of each was dissected out and placed in turn between two micro-electrodes.

During larval development the average mid-gut pH increased reaching a maximum two days before the prepupal stage. The highest averave pH of fifth-instar larvae was observed in the virus-free stock followed, in decreasing order, by the Dutch, French and cheiranthi.

A characteristic (distinctive) pH pattern was observed in the alimentary canal in all larvae ie. slightly alkaline in the crop, gradually increasing up to the central mid-gut and decreasing after this point.

GV suspensions which had been subjected to a range of pHs of glycine/NaOH buffer and gut fluids were bioassayed

with second-instar larvae. Considerable losses of viability, depending on the pH, were observed. Alkaline dissolution of the capsules studied by optical density measurements showed that carbonate buffers were more effective in dissolving the capsules than borate buffers at similar pHs.

Observations with the electron microscope showed that gut fluid degraded the capsules and virions almost completely, and that buffers of comparable pH were less effective.

GV was fed to fifth-instar P. brassicae, Lacanobia oleracea and Spodoptera littoralis, recovered, and then bioassayed with second-instar virus-free P. brassicae. The results showed a high inactivation of the virus in the mid-gut, particularly of P. brassicae.

GV fed to second-instar virus-free larvae on acidified diet resulted in a higher mortality than GV fed on standard diet. On the other hand, addition of proteolytic enzyme inactivators apparently had no effect on mortality either beacause they were destroyed in the gut or the proteases had no effect on virus infection.

Measurements of the surface-volume ratio of the mid-gut decreased only slightly with larval instar (about eight times at maximum) and was insufficient to account for the marked increase in resistance to GV observed which was of the order of five log.

Microscopic sections of the peritrophic membrane showed that it originates from a ring of mid-gut cells situated in the anular pocket formed by the projection of the fore-gut into the mid-gut. Further layers were delaminated from the brush border along the mid-gut. The time of appearance of the peritrophic membrane coincided with the hatching of the larvae.

ADDENDUM

Composition and preparation of the semi-synthetic diet used in rearing P. brassicae larvae, as described by David and Gardiner (1966a).

(a)	Distilled water	110 ml
	Potassium hydroxide (4 M sol.)	1.8 ml
	Casein (light white soluble)	12.6 g
(b)	Sucrose	12.6 g
	Wheat germ (Bemax)	10.8 g
	Cabbage leaf (dried powder)	5.4 g
	Salt mixture	3.6 g
	Whatman chromedia cellulose powder, CF 11 grade	1.8 g
(c)	Choline chloride (10% soln.)	3.6 ml
	Methyl paraphydroxy-benzoate (15% in 95% EtOH)	3.6 ml
	Formaldehyde soln. (10% w/v)	1.5 ml
	Vitamin stock	0.6 ml
(d)	Distilled water	200 ml
	Agar (fine Japanese powder)	9 g
(e)	1-Ascorbic Acid	1.5 g
	Aureomycin (veterinary grade)	0.8 g

The composition of the salt mixture (in g.) is : $CaCO_3$, 120 ; K_2HPO_4; 129 ; $CaPHO_4 . 2H_2O$, 30 ; $MgSO_4 . 7H_2O$, 40.8 ; NaCl, 67 ; $FeC_6H_5O_7 . 6H_2O$, 11 ; KI, 0.32 ; $MnSO_4 . 4H_2O$, 2.0 ; $ZnCl_2$, 0.10 ; $CuSO_4 . 5H_2O$, 0.12.

The composition of the vitamin stock (in mg) is : nicotinic acid, 600 ; calcium pantothenate, 600 ; riboflavine (B_2), 300 ; aneurine hydrochloride (B_1), 150 ; pyridoxine hydrochloride (B_6), 150 ; folic acid, 150 ; D-biotin, 12 ; cyanocobalamine (B_{12}), 1.2 ; 100 ml water.

The "cabbage leaf" powder was prepared by drying the large leaves of kale, broccoli, brussels sprouts, or the outer, green leaves of cabbages. Before the leaves were dried, the main ribs were removed. For small quantities the leaves may be dried for 15 - 20 minutes, in a single layer, on the wire mesh shelves in a laboratory oven at 100-105°C. The door should be opened once or twice to let out the steam. Afterwards, the leaves may be crushed up roughly by hand and ground under a roller or in an electric blender type coffee mill. Finally the powder is sieved through a 60-mesh sieve. Larger quantities can be best treated in a ventilated drying oven afterwards ground up in a small Christy-Norris type mill fitted with a 0.5 mm mesh screen.

The veterinary grade aureomycin soluble powder (Cyanamid of Great Britain) contained 25g. of chlortetracycline hydrochloride per pound.

The diet was made up in an M.S.E. blender with a capacity of 800 ml. The ingredients listed in (a) are first thoroughly blended together until a thick cream is formed ; the solids (b) are then added, with further blend-

ing. The solutions (c) are next added, separately, while the blender is running. Meanwhile the agar solution (d) has been prepared in a boiling water bath. It is cooled to 70°C and added to the mixture. Finally the ingredients (e) are added, and the whole diet is thoroughly blended. The formula as given yields about 379 g. of finished diet.

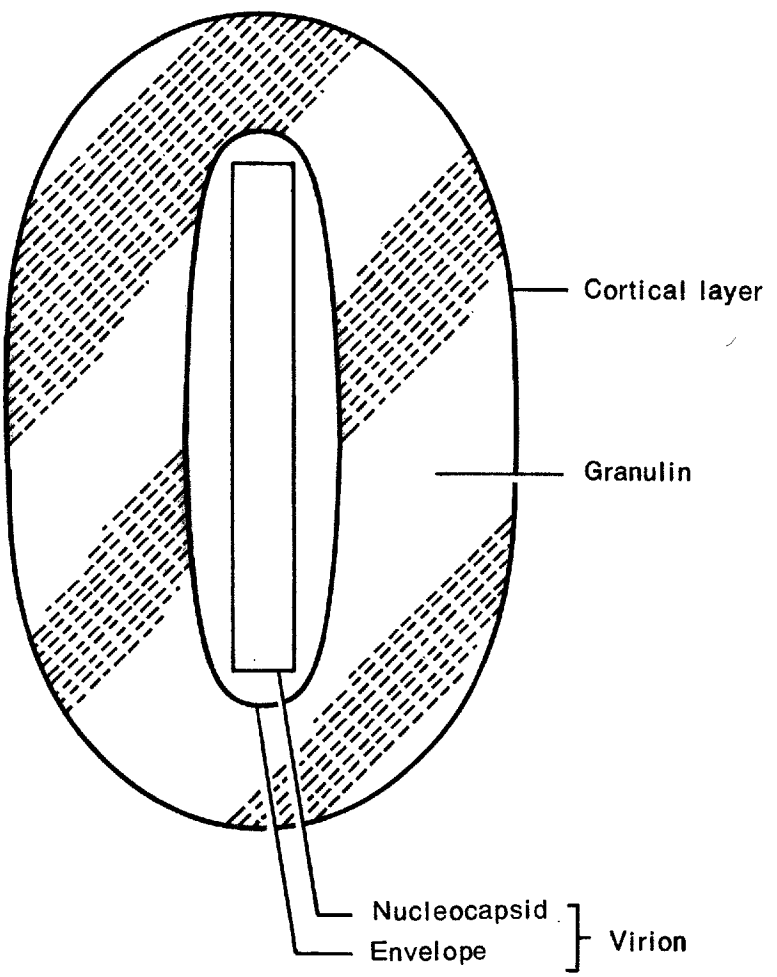


Fig. 42

Diagrammatic cross-section of a granulosis virus capsule.

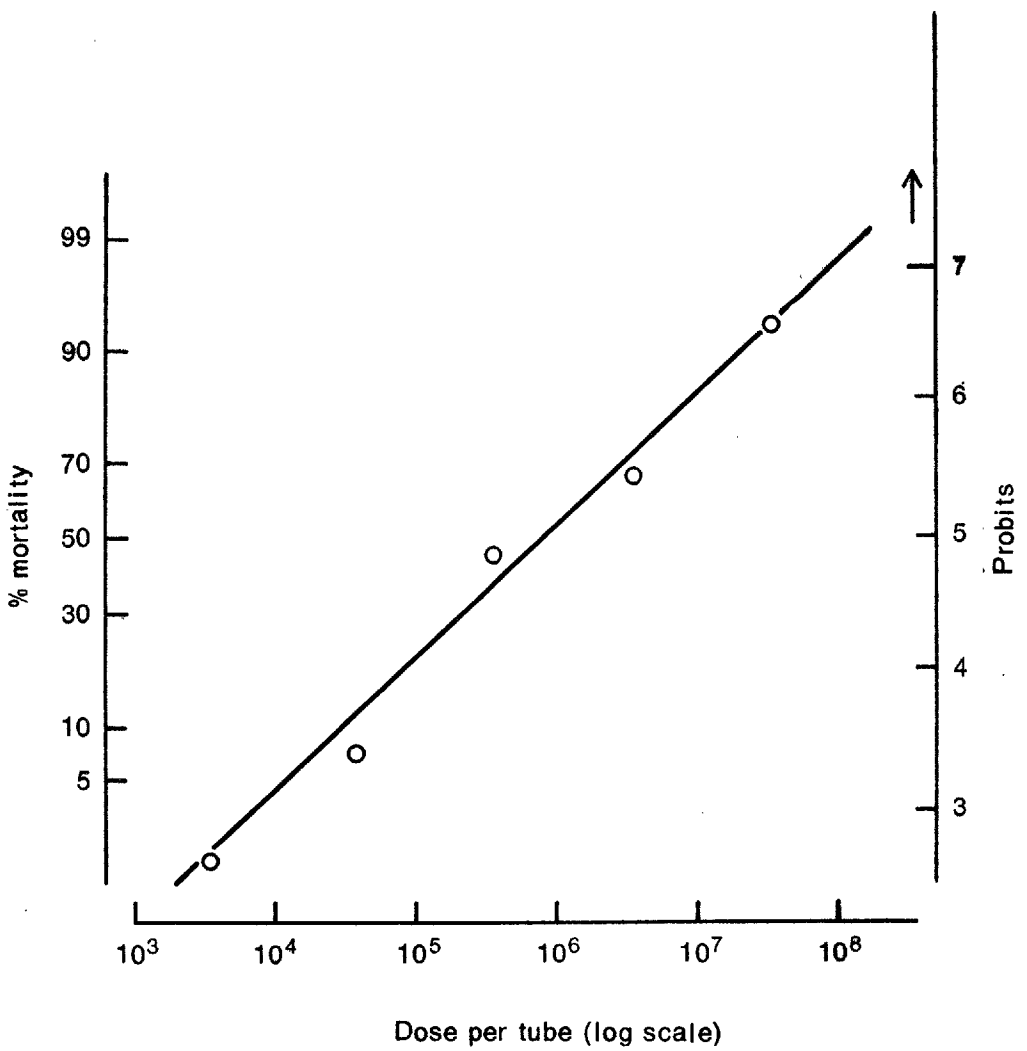


Fig. 43

Dosage-mortality response of second-instar virus-free *P. brassicae* larvae on formalin-free diet. Arrow indicates 100% mortality.

TABLE 25

Probit analysis for three individual assays of third-instar virus-free larvae of *P. brassicae* fed with granulosis virus.

Test No.	LD_{50} Capsules/larva	SE LD_{50}	95% fiducial limits for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 for heterogeneity	No. larvae in test
1	1.7×10^6	1.2×10^3	1.0×10^6-2.6×10^6	1.65	0.28	0.98	108
2	2.1×10^6	1.1×10^3	1.4×10^6-3.0×10^6	1.62	0.25	0.03	155
3	3.3×10^6	1.1×10^3	2.3×10^6-4.5×10^6	1.58	0.24	0.02	173

TABLE 26

Probit analysis for six individual assays of fourth-instar virus-free larvae of *P. brassicae* fed with granulosis virus.

Test No.	LD_{50} Capsules/larva	SE LD_{50}	95% fiducial limits for LD_{50} capsules/larva	Slope b	SE of the slope	χ^2 for heterogeneity	No. larvae in test
1	5.2×10^7	1.3×10^5	2.9×10^7 - 9.4×10^7	1.30	0.192	4.7	114
2	6.4×10^7	2.0×10^5	1×10^7 - 5.3×10^7	1.06	0.163	10.6	118
3	7.2×10^7	1.2×10^5	4.3×10^7 -12 $\times 10^7$	1.66	0.272	1.4	117
4	4.3×10^7	1.2×10^5	2.6×10^7 - 7.1×10^7	1.66	0.274	1.0	118
5	5.4×10^7	1.2×10^5	3.2×10^7 - 9.0×10^7	1.60	0.259	1.4	118
6	11 $\times 10^7$	1.2×10^5	6.5×10^7 -19 $\times 10^7$	1.44	0.221	0.7	120

TABLE 27

Average pH of the contents of each of eight sections of the gut of fourth-instar virus-free *P. brassicae* larvae at three different ages.

	Gut sections examined							
	Th_1	Th_3	Ab_1	Ab_2	Ab_2	Ab4	Ab_5	Ab_6
Age of larvae	24 hours							
Mean pH	7.49	8.00	8.29	8.60	8.90	8.76	8.42	7.81
S.D.	0.50	0.4	0.14	0.50	0.28	0.18	0.31	0.26
No. of observations	5	3	6	5	6	6	6	5
Age of larvae	48 hours							
Mean pH	7.84	8.89	9.18	9.07	9.6	9.76	9.41	8.42
S.D.	0.79	0.42	0.53	0.29	0.32	0.44	0.23	0.33
No. of observations	5	4	5	6	6	5	4	4
Age of larvae	72 hours							
Mean pH	7.54	8.56	8.80	9.16	9.64	9.79	9.61	8.90
S.D.	0.48	0.34	0.40	0.23	0.14	0.27	0.39	0.21
No. of observations	8	7	8	8	8	8	8	8

TABLE 28

Average pH of the regurgitated fluid and of the contents of each of nine sections of the gut of fifth instar virus-free P. brassicae larvae at six different ages.

	Regurgitate	Gut sections examined								
		Th_1	Th_2	Th_3	Ab_1	Ab_2	Ab_3	Ab_4	Ab_5	Ab_6
Age of larvae		4 hours								
Mean pH	-	9.15	8.83	9.62	9.31	9.38	9.55	9.75	9.21	8.82
S.D.	-	0.67	0.53	0.29	0.25	0.56	0.72	0.27	0.46	0.34
No. of observations	-	6	6	5	3	5	6	6	6	6
Age of larvae		24 hours								
Mean pH	8.91	8.35	8.73	9.10	9.32	9.50	9.71	9.87	9.79	9.04
S.D.	0.77	0.63	0.54	0.57	0.27	0.16	0.30	0.16	0.16	0.25
No. of observations	7	7	7	7	7	7	7	7	7	7
Age of larvae		48 hours								
Mean pH	9.55	8.81	9.20	9.58	9.56	9.72	10.07	10.10	0.82	8.77
S.D.	0.29	0.10	0.41	0.30	0.32	0.38	0.13	0.11	0.36	0.28
No. of observations	7	7	7	7	7	7	7	7	7	7

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TABLE 28 (continued)

Average pH of the regurgitated fluid and of the contents of each of nine sections of the gut of fifth-instar virus-free P. brassicae larvae at six different ages.

	Regurgitate	Gut sections examined								
		Th_1	Th_2	Th_3	Ab_1	Ab_2	Ab_3	Ab_4	Ab_5	Ab_6
Age of larvae		72 hours								
Mean pH	8.81	8.56	8.78	9.29	9.52	9.86	10.03	10.25	9.90	9.11
S.D.	0.78	0.79	0.49	0.47	0.41	0.25	0.24	0.14	0.17	0.24
No. of observations	10	11	11	11	11	11	11	11	11	11
Age of larvae		96 hours								
Mean pH	9.19	8.66	9.16	9.75	10.06	10.30	10.46	10.43	10.08	9.25
S.D.	0.80	0.80	0.47	0.11	0.14	0.14	0.23	0.20	0.19	0.34
No. of observations	7	7	7	7	7	7	7	7	7	6
Age of larvae		120 hours								
Mean pH	8.88	8.50	8.83	9.36	9.72	10.01	10.12	10.31	10.09	9.31
S.D.	0.85	0.73	0.59	0.46	0.24	0.25	0.28	0.22	0.25	0.37
No. of observations	5	7	7	7	7	7	7	7	7	7

TABLE 29

Average pH of the reguritated fluid and of the contents of each of nine sections of the gut of four stocks of P. brassicae fifth-instar larvae 72 hours old.

	Regurgitate	Gut sections examined								
		Th_1	Th_2	Th_3	Ab_1	Ab_2	Ab_3	Ab_4	Ab_5	Ab_6
Stock					cheiranthi					
Mean pH	8.44	7.86	8.13	8.65	8.94	9.10	9.59	9.79	9.45	8.59
S.D.	0.76	0.76	0.68	0.43	0.33	0.57	0.31	0.23	0.31	0.33
No. of observations	25	27	26	26	27	27	27	27	27	27
Stock					French					
Mean pH	8.73	8.03	8.24	8.84	9.16	9.43	9.71	9.80	9.50	8.81
S.D.	0.74	0.79	0.59	0.47	0.44	0.30	0.26	0.29	0.37	0.38
No. of observations	22	22	22	23	23	23	23	23	23	23
Stock					Dutch					
Mean pH	8.81	8.13	8.57	9.10	9.42	9.68	9.96	10.05	9.57	8.76
S.D.	1.04	0.89	0.73	0.68	0.53	0.40	0.32	0.28	0.29	0.36
No. of observations	21	22	22	23	23	23	23	23	23	23
Stock					Virus-free					
Mean pH	8.81	8.56	8.78	9.29	9.52	9.86	10.03	1-.25	9.90	9.11
S.D.	0.78 0	0.79	0.49	0.47	0.41	0.25	0.24	0.14	0.17	0.24
No. of	10	11	11	11	11	11	11	11	11	11

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