VIRUS INFECTION IN LEPIDOPTERA WITH SPECIAL
REFERENCE TO A NUCLEAR POLYHEDROSIS OF
Galleria mellonella L.

by

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# TABLE OF CONTENTS

I. INTRODUCTION  
II. REVIEW OF LITERATURE  
A. NATURAL COURSE OF VIRUS DISEASE  
B. MECHANISMS OF RESISTANCE TO VIRUS INFECTIONS  
C. ESTIMATING VIRUS ACTIVITY  
III. METHODS  
A. REARING TEST INSECTS  
B. ISOLATION OF POLYHEDRA AND PREPARATION OF VIRUS PARTICLE SUSPENSIONS  
C. INITIATION OF INFECTION  
D. DIAGNOSIS OF DISEASE  
E. DESIGN AND ANALYSIS OF INFECTION TESTS  
IV. RESULTS  
A. RESPONSE FOLLOWING INFECTION OF FOURTH-INSTAR LARVAE  
1. Response to Ingested Virus  
2. Response to Intralymphal Administration  
3. Comparison of Responses  
B. EFFECTS OF METAMORPHOSIS ON INFECTION  
1. Changes in Resistance as Larvae Approach Pupation  
2. Mortality of Pupae and Adults Following the Ingestion of Polyhedra by Late Larvae.  
3. Susceptibility of Pupae and Adults to Intralymphal Administration of Virus Particles  
C. FATE OF INGESTED VIRUS  
1. Dissolution of Polyhedra  
2. Excretion of Virus Particles  
3. Inactivation of Virus Particles by the Midgut Fluid  
4. Effects of Alkaline pH on Polyhedra and Virus Particles  
5. pH of Midgut Fluid  
6. Possible Phagocytosis of Virus Particles  
   a. Reaction to India Ink and Trypan Blue  
   b. Infection by Virus Particles Following Blockage of the Phagocytes  
7. Vaccination with Live Virus  
V. DISCUSSION  
VI. SUMMARY  
VII. ACKNOWLEDGEMENTS  
VIII. REFERENCES
I. INTRODUCTION

Insects like so many other living forms are susceptible to the infectious agents called viruses. Four types of insect viruses are recognized and these are: the nuclear polyhedroses (Borrelinavirus), the cytoplasmic polyhedroses (Smithiavirus), the granuloses (Bergoldivirus), and non-inclusion viruses (Moratorvirus).

Nuclear polyhedrosis viruses are rod-shaped, about 200 to 400 μ in length and 20 to 70 μ in diameter (Bergold, 1958). The virus particles are occluded by protein that crystallizes to form the polyhedral inclusion bodies which may range in size from 1.0μ to 10.0μ. Particles and inclusion bodies are formed in the nuclei of susceptible cells. These viruses have been isolated from various Lepidoptera and sawflies and from a dipteran, Tipula paludosa (Meig.).

Granulosis viruses are formed in the same manner as the polyhedrosis viruses (Dr. F.T. Bird, personal communication). Rapid disintegration of the nuclear membrane led to the belief that the virus particles are formed in the cytoplasm (Bird, 1958), but essentially they are nuclear infections. They differ from the nuclear polyhedroses in that each inclusion body is about 0.2μ by 0.5μ and contains only one virus particle (Bergold, 1948; Bird, 1958; Martignoni, 1957; Tanada, 1953). This type of disease has been found only among Lepidoptera.

The cytoplasmic polyhedroses are characterized by the formation of polyhedral-shaped crystals in the cytoplasm of the midgut cells of Lepidoptera. The crystals are protein and spherical particles are occluded within them. The particles range in size from 15 μ to 80 μ and the polyhedra are from 0.5μ to 15μ depending on the host species.
(Smith, in Burnet and Stanley, 1959). The infectious nature of the particles has not been established but when intact polyhedra are fed to larvae typical infection occurs which results in the death of the larva (Bird and Whalen, 1954).

Of the Moratorvirus group, Tipula iridescent virus is best known. This virus was first isolated from Tipula paludosa (Meig.) (Xeros, 1954) but in the laboratory it appears to develop in a wide range of hosts (Smith and Rivers, 1959, Bird, 1960). The particles are about 130 μ in diameter and have 20 sides (Smith and Williams, 1958). They also appear to form in the cytoplasm of susceptible cells but like the granuloses, virogenesis probably takes place in the nuclear material (Dr. F.T. Bird, personal communication).

The present study is concerned with the host-parasite relationships of nuclear polyhedroses in Lepidoptera. These diseases have been observed in populations of Bombyx mori L., Porthetria dispar L. and Lymantria monocha L. for more than half a century (Bolle, 1898; Glaser, 1915; Paillot, 1933) and in the last 20 years they have been found in many other species of Lepidoptera (Hughes, 1957). Their structure, chemistry and pathology have been investigated (Bergold, 1958) and in some cases they have been used to control insect infestations (Steinhaus and Thompson, 1949 a, b; Thompson, 1951; Hofmaster and Ditman, 1961; Stairs and Bird, 1962). These viruses vary widely in their pathogenicity and only the highly pathogenic are useful in biological control. Some of the less pathogenic species, however, may become useful when basic host-pathogen reactions are understood.
The wax moth, *Galleria mellonella* L. and its specific nuclear polyhedrosis virus was used as a model test system to obtain basic information on host-pathogen interactions. However, in some experiments *Bombyx mori* L. and *Choristoneura fumiferana* (Clemens) and their respective nuclear polyhedrosis viruses were used. Methods were developed to determine quantitatively the response of the host population to virus over a wide range of dosages administered orally and intralymphally. Changes in response that occurred during the late larval stage and during metamorphosis were investigated. Also, the mechanisms by which individuals prevent or overcome infections were studied.
II. REVIEW OF LITERATURE

A. NATURAL COURSE OF VIRUS DISEASE

Virus disease in warm-blooded animals appears to develop in a step-wise manner. Penetration of a susceptible cell is followed by multiplication and then liberation of more infective virus (Burnet, 1960). The newly produced virus either infects adjacent cells or is spread to other susceptible internal organs where further multiplication occurs. Primary infections are located in the respiratory or intestinal membranes or the skin. Generalized infection may occur also in these membranes but it is often found in the glands, lymph nodes, brain and spinal cord, lungs, liver or spleen. The frequency of symptomatic infection varies from 100% for small pox and measles to less than 1% in poliomyelitis (Burnet, 1960). Also, it has been shown recently that a large number of human adenoviruses multiply and survive in the respiratory and alimentary tracts without causing symptomatic infections at all (Heubner, in Pollard, 1959).

The most complete account of the events in pathogenesis of a natural virus disease in a warm-blooded animal is that given by Fenner (1949) for infectious ectromelia of mice (mouse-pox) and there is much evidence which suggests that the conclusions reached concerning this disease may be directly applicable to smallpox and other human exanthems caused by virus (Burnet, 1960). The sequence of events in mouse-pox follows a constant pattern through which the virus passes in a step-wise manner. The virus enters through a small abrasion and the first cycle of infection occurs in the surface layers of the skin. In subsequent cycles virus
reaches the lymph nodes and from here it is spread to the bloodstream. The phagocytic cells of the liver and spleen then ingest virus particles and the same cycle proceeds in these organs.

After an interval of at least a day more virus is released into the bloodstream and a secondary viremia occurs in the skin. The secondary rash appears during the eighth or ninth days while the primary lesion is evident seven days after primary infection. Fenner also titrated the virus in the infected organs at time intervals and established that all virus multiplication had occurred during the incubation period, that is, before the appearance of the primary lesion. The degree of viremia appears to be directly related to the amount of virus multiplication that occurs in the liver and spleen. In acute fatal cases the virus content of these organs is high but not enough time has elapsed for skin lesions to develop. Individuals that survive the disease possess a very high degree of immunity but this immunity is not transmitted to their progeny.

Insect viruses also appear to develop in a step-wise manner. Penetration of susceptible cells is followed by periods of multiplication and liberation (Bird, 1959; Aizawa, 1959) but the sequence of spread from one organ to another is not known. In sawflies polyhedrosis viruses attack the midgut cells only and infection spreads from primary foci to all functional gut cells (Bird, 1949). In Lepidoptera suffering from nuclear polyhedrosis viruses a number of organs are attacked. Generally, evidence of infection appears first in the tracheal epithelium and subsequently in the blood, fat and epidermis (Glaser, 1915; Paillot, 1933; Bird and Whalen, 1954). Granulosis viruses also follow the same course of infection (Bird, 1958; Tanada, 1959).

The events that occur during the interval between the consumption of
contaminated food and the manifestation of disease in susceptible tissues are unknown. Histological changes in susceptible cells have been described (Paillot, 1933; Heidenreich, 1940; Komárek and Breindl, 1924; Bird, 1949, 1953, 1957; Bird and Whalen, 1953, 1954) but in view of the studies on animal viruses (Fenner, 1949) it is probable that all virus multiplication has occurred in a cell by the time its structure is changed histologically. Aizawa's (1959) investigations on the multiplication of silkworm nuclear polyhedrosis virus provide evidence that this is true for insects. In these studies virus titer reached a maximum at 43 hours and remained high. The first cytological changes are not observed until the third day and polyhedra do not appear until the fifth day (Paillot, 1933). Cells become filled with polyhedra and the larva dies on the sixth or seventh day.

B. MECHANISMS OF RESISTANCE TO VIRUS INFECTIONS

Once a virus has initiated an infection in a warm-blooded animal, certain steps are taken to eliminate it. In individuals that have had no previous infection only generalized mechanisms act against the virus immediately. These non-specific factors have little therapeutic value, hence they have not received as much attention as antibody reactions. Recently, however, interest in non-specific reactions, such as body temperature, hormones, etc., has been renewed.

Body temperature and hormones appear to affect the survival of virus. High temperature simply inactivates viruses (Smorodintsev, 1960), but the effects of hormones are more complex. For example, estrogen in-
creases resistance while cortisone increases susceptibility (Shilo, 1959).

Since the discovery of the properdin system (Pillemer et al., 1954), attention has been focused on non-specific inhibiting factors in normal serum. The action of the properdin system is dependent on the presence of complement-fixing antigen, Mg++, and properdin in the serum. When all three are present virus may be inactivated. The exact nature of the mechanism is not known but the absence of one of the components renders the system ineffective. Properdin itself is a heat-labile, heterogeneous globulin type of protein that may be absorbed from serum by zymosan, an insoluble polysaccharide from the walls of certain fungal cells.

Further studies have shown that zymosan adsorbs another inhibiting factor that differs in many respects from properdin (Smorodintsev, 1960) and it is possible that the original tests were made with a mixture of these inhibitors. This new factor is present in large amounts in animals which have small amounts of properdin and vice-versa. It has been identified as β-lipoprotein. It is more heat-stable than properdin but perhaps the most important difference is that the presence of complement-fixing antigen and Mg++ are not necessary for its action.

Isaacs and Lindenmann (1957) discovered a substance that inhibits the multiplication of a wide range of viruses. Cells that have been infected with one kind of virus produce this substance called interferon, which prevents the multiplication of other kinds of virus in the same cells. Interferon does not act directly on the virus nor does it affect virus adsorption by the cell but retards virus multiplication within the cell. According to Burke and Isaacs (1958), interferon is not produced if the cell metabolism has been fully converted to the production of virus. Along the same line, it has been found that certain cells that are chron-
ically infected with virus but maintain normal morphological structure are resistant to other viruses possessing cytopathic activity (Henle et al., 1959; Ho and Enders, 1959).

The role of phagocytes in the destruction of virus has not been studied extensively because virus particles are so small that they cannot be seen under the light microscope and special techniques utilizing electron microscopy or fluorescent-antibody must be used to detect them. Although Baddour et al. (1953) have demonstrated phagocytosis of an influenza virus by polymorphonuclear leucocytes, in vitro experiments have shown that most viruses are not readily phagocytised (Sabin, 1935; Smorodintsev, 1960). Burnet (1960) concluded that it is probably reasonable to assume that phagocytosis plays no major part in defense against virus infections. However, this statement is based on a limited number of observations and the literature indicates that more information about the action of phagocytes on viruses may be obtained by employing specially developed techniques.

The foregoing are the more generalized reactions and from a therapeutic point of view are not as important as the specific reactions to be discussed in the next paragraphs.

It is well known that humans who have survived a virus infection such as smallpox or measles are usually immune to subsequent attack by the same virus. During the course of the disease a substance that inactivates the virus is produced and released into the bloodstream. This substance is known as antibody and its titer often remains high enough to provide protection from the virus over extended periods of time, sometimes for life (Burnet, 1960). This is the chief means by which virus diseases are overcome.
In many cases even a subclinical infection will cause the production of antibody and the individual will be protected against subsequent large doses of virus. Immunologists have used this principle to advantage, causing artificial subclinical infections following which the individual develops an immunity. Live viruses will cause the strongest antibody response but viruses that have been killed by heat, ultraviolet light or formaldehyde may also induce a significant response (Gard. and Maaloe, in Burnett and Stanley, 1959; Polley, 1959).

When the antigenic stimulus of a virus is intense many clones of specific antibody-producing cells proliferate throughout the body and these may be maintained over long periods, producing antibody continuously and providing long-term protection. According to Burnet (1960) and Fenner (1949), new infections are probably cut off as soon as the virus enters the blood stream in which the specific antibodies are circulating.

Comparatively little is known about mechanisms of resistance to virus infections in insects. In Lepidoptera the high alkalinity of the mid-gut fluid is believed to inactivate virus (Bergold, 1958), and recently Aizawa (1962) reported inactivation in vitro of the nuclear polyhedrosis virus of Bombyx mori L. by midgut fluid from larvae. The active substance was effective at neutral pH and Aizawa concluded that it is probably an enzyme. This is the only report of an antiviral substance in insects.

Once an infection has been initiated the virus spreads to all susceptible tissues where it multiplies and finally causes the production of characteristic inclusion bodies and death of the insect. The only exception is that discovered by Bird (1949, 1953) for the sawfly, Diprion
hercyniae (Htg.), in which the regenerative cells of the susceptible midgut are resistant to attack by virus. At metamorphosis the regenerative cells proliferate a new gut that is resistant to infection. Infection does resume, however, when the cells become functional but the delay gives the insect opportunity to complete its development to the adult stage. The adult behaves as a normal individual except that it transmits virus to a fraction of its progeny which succumb to disease in the early larval instars. This method of overcoming a virus infection is mutually beneficial to both host and parasite, insuring the survival of each. Lepidoptera may cope with virus infections in a similar manner but this has not been demonstrated.

The effects of blood serum and phagocytes on virus infections in insects have not been investigated. Antibodies such as those found in warm-blooded animals have not been demonstrated although a number of attempts have been made to induce their production and detect their presence (Briggs, 1958; Stephens, 1959; Paillot, 1933; Glaser, 1918, 1925; Cameron, 1934; Huff, 1940). Antibacterial substances are known (Frings et al., 1948; Briggs, 1958) but their effects on viruses has not been studied.

Immune responses to bacteria have been observed among insects (Meta

alnikov, 1920; Paillot, 1920; Glaser, 1918, 1925; Stephens, 1959) but true antibodies have not been demonstrated. Metalnikov (1920) showed that blood cells actively phagocytised bacteria and he maintained that they could be sensitized so that an immune response would be observed. Paillot (1920) insisted that a humoral reaction took place before phagocytosis. Recently, Briggs (1958) studied the humoral reactions of insect haemolymph and concluded that antibodies are not produced. Stephens (1959) reached
the same conclusion and showed that serum proteins function in immunity but true antibody is not produced.
C. ESTIMATING VIRUS ACTIVITY

Titrations of virus activity may be relative or absolute. A relative titration expresses the activity of a particular suspension in relation to that of another, whereas an absolute titration attempts to express activity in reference to a number of particles or weight of some chemical. The ultimate goal is to assess the effects of single particles.

Particle counts can be made directly with the aid of the electron microscope. The method most frequently employed is known as the "spray-droplet method" (Backus and Williams, 1950) in which a small amount of suspension is nebulized onto a thin film and the particles are counted directly under the microscope. A known quantity of latex spheres is mixed with the suspension and the concentration of virus is estimated in reference to them. A modification of this technique known as "agar-gel infiltration" has also been frequently employed (Kellenberger and Arber, 1957). In this method the suspension of virus and latex particles is placed over an agar-gel but separated from it by a collodion membrane. The liquid medium diffuses through the membrane and into the agar leaving the particles on the collodion. The films are then fixed over formol and collected on grids for examination under the electron microscope.

A third method, the "sedimentation" technique, employs the same principle but the particles are deposited on the collodion by centrifugation. This method, however, is not in general use because it is considered too complex and tedious for routine work (Schwerdt, cited in Burnet and Stanley, 1959).
Indirect methods of estimating virus concentration have been developed but there are numerous objections to their use. The main objection is that they are usually based on a number of assumptions that are difficult to resolve, e.g. virus purity, spherical particle shape, etc. Estimates based on the number of haemagglutinating particles are rapidly and easily obtained but are applicable only to those viruses that are able to agglutinate red cells (e.g. influenza virus) (Isaacs, 1957). This is a very convenient titration technique but certain difficulties in relating the haemagglutinating ability to infectivity have been encountered (Burnet, 1960).

Virus suspensions may be titrated by two methods. If a single layer of susceptible cells is available, a small quantity of suspension may be spread over it and, after a period of incubation, small plaques of destroyed cells appear where single plaque-forming units (PFU) have succeeded in causing infection (Luria et al, 1951). One bacteriophage particle is capable of initiating a plaque but 2 to 20,000 animal virus particles may be necessary to initiate the formation of a plaque (Schwerdt, cited in Burnet and Stanley, 1959). The number of plaques or lesions is directly related to the dosage of virus. This is a most accurate and rapid method and may be used wherever a monolayer of cells is available, e.g. skin of an animal, surface of a leaf, or a monolayer tissue culture.

Viruses may also be titrated in living animals. Aliquots of the virus suspension are given to susceptible animals by some route that causes infection or death. In order to obtain satisfactory results each concentration must be tested against a number of animals, some of which will respond to the virus. The number responding increases with dosage. The dosage which causes 50% of the test animals to respond is known as the
median effective dosage (Isaacs, 1957). The mathematical accuracy of the median effective dosage (E.D. 50) can be calculated (Finney, 1952) and comparisons of relative potency, similarity of response, etc., can be made.

Only indirect methods of counting insect virus particles have been used. Bergold (1943, 1947, 1951) tried to relate protein content to infectivity and others have used counts of inclusion bodies (Bird and Whalen, 1953; Martignoni, 1957; Martignoni and Schmid, 1961). Relative titrations have also been made (Aizawa, 1953, 1959; Bird, 1959).

The actual number of particles may be estimated from polyhedral body counts. The particles are contained, in bundles of 1 to 15, within the polyhedral bodies (Bergold, 1947, 1951; Bird, 1949, 1957; Morgan et al., 1955) and the average number of bundles per body can be determined by dissolving them in weak alkali solutions and counting the released bundles under the electron microscope (Hughes, 1953). To obtain the average number of particles per bundle thin sections of inclusion bodies can be made and the particles can be counted by using the electron microscope (Morgan et al., 1955; Bird, 1957; Bergold and Suter, 1959). This is probably the best method of estimating the number of particles per bundle and by combining this with the number of bundles per inclusion body and the number of inclusion bodies per unit volume an estimate of the number of particles per unit volume may be obtained. Accurate counts of insect virus particles will also be obtained when the "latex" methods described above are employed.

Some progress has been made in culturing insect tissues (Wyatt, 1956; Grace, 1958; Day and Grace, 1959; Aizawa and Vago, 1959; Jones and Cunningham, 1960) and cultures have been obtained which were susceptible to
viruses (Trager, 1935; Martignoni and Scallion, 1961). In one case tissue cultures have been used for virus assay (Vaughn and Faulkner, in press). The response of living insects has been used to titrate nuclear polyhedrosis viruses of B. mori and Choristoneura fumiferana (Clemens) (Bergold, 1947, 1951). Similarly, Aizawa (1959) used this approach to study certain aspects of nuclear polyhedrosis virus infections in the silkworm, B. mori. Bird and Whalen (1953) estimated the median lethal dosage (LD50) for a virus of the European pine sawfly, Neodiprion sertifer (Geoffr.), and Martignoni (1957) did the same for a granulosis virus of Zeiraphera (Fucoidea) griseana (Hubner), the larch budmoth. Bucher (1956) presented data on the response of the forest tent caterpillar, Malacosoma disstria (Hubner), to a nuclear polyhedrosis virus and discussed dosage effect in some detail. He pointed out that the median lethal dose does not fully describe the response of a host population to a virus and observed that when dosage-response curves were transformed to straight lines the slopes were low in comparison to those obtained for insecticides. This was interpreted as meaning that there is a much wider range of resistance (or susceptibility) to virus.

If host response is studied over a wide range of dosages valuable information about the host-parasite interaction may be obtained. Martignoni (1957) employed this principle while investigating a granulosis virus of Z. griseana and claimed that populations became less variable and more resistant in response to virus as the epizootic proceeded. Similar studies have shown that different populations of the California oakworm, Phyryganidia californica Packard, may vary in their response to infection by a nuclear polyhedrosis virus (Martignoni and Schmid, 1961).
III. METHODS

A. REARING TEST INSECTS

A population of 8,000 to 10,000 wax moth larvae was maintained throughout this study and all stages were reared in one-gallon jars (Fig. 1). The jars were fitted with screen tops and kept at 30 ± 1°C and at a relative humidity of about 40 per cent. About 500 mature larvae were reared in one jar on a diet prepared from Pablum cereal (460 gm), honey (100 ml), corn meal (45 gm), glycerine (100 ml) and baker's yeast (8 gm). The glycerine, corn meal, honey and yeast were mixed thoroughly and the cereal was added, with continued mixing, until the mixture reached the consistency of wet sand. Fresh food was prepared twice weekly and stored at 4°C.

The wax moth completes a generation in about two months. The larval stage lasts about 30 days with four to five days being spent in each of the first four instars and nine to ten in the last instar. The pupal stage lasts about 10 days. The adult lives about 10 days but eggs may be laid within a day after eclosion. These hatch nine to ten days after they are deposited.

Larvae and pupae of B. mori and C. fumiferana were supplied from the quarantine rearing department of the Insect Pathology Research...
Fig. 1. Various stages of *Galleria mellonella* L. in the rearing jars. 0.25X.
Institute by Miss A.R. McMorrnan. They were reared on their respective
host plants, mulberry and balsam fir buds.

In all tests, wax moth and budworm larvae were confined separately
in small petri dishes (50mm x 15 mm, Fig. 2). Silkworms were placed in
larger dishes (100mm x 60mm). Appropriate food was provided until the
larvae ceased to feed.

B. ISOLATION OF POLYHEDRA AND PREPARATION OF
VIRUS PARTICLE SUSPENSIONS

Polyhedra were obtained by allowing infected tissues to rot in
water at room temperature for several weeks. The polyhedra, freed from
the tissue by putrefaction, settled to the bottom of the container where
they appeared as a whitish sediment. This sediment, which also contain-
ed tissue remnants and bacteria, was purified by differential centri-
fugation. Pure suspensions of polyhedra were stored in water at 4°C.
Growth of bacteria was prevented by the addition of 140 μgm of penicillin
and 200 μgm streptomycin per ml of the polyhedral suspension. The
number of polyhedra in stock suspensions were counted using a standard
Petroff-Hauser bacteria counting chamber. As the polyhedra are heavy
and tend to settle rapidly the suspensions were mixed well before each
sample was taken.

In some experiments it was necessary to inject virus particles into
the body cavity to initiate infection. Polyhedra may be dissolved by
using weak alkali solutions after the method outlined by Bergold (1953).
In this method about 200 volumes of sodium carbonate (0.005M - 0.006M)
is added to dissolve the inclusion body protein. The virus particles are
Fig. 2. A fifth-instar *G. mellonella* larva in a small petri dish. 2X.
not affected by this treatment. The particular experimental conditions used were as follows:

1. One ml of a suspension containing from $2 \times 10^3$ to $5 \times 10^8$ polyhedra per ml was centrifuged for ten minutes at 3000g to concentrate the polyhedra in a pellet at the bottom of the tube and the supernatant was discarded.

2. The pellet was resuspended in 0.9 ml of distilled water, 1.0 ml of 0.1M sodium chloride and 0.1 ml of 0.1M sodium carbonate (making a total of 2.0 ml) and placed on a slow shaker (40 oscillations per min.) for one hour at room temperature.

3. Undissolved protein and other large particles were removed by centrifugation at 3000g for 15 minutes and the supernatant was recentrifuged at 12,000g for one hour to concentrate the released virus particles in a pellet at the bottom of the tube. The supernatant was discarded.

4. The small bluish-white pellet of virus was resuspended in 1.0 ml of distilled water. This suspension was stored in the dark at 4°C. It contained the virus particles from $2 \times 10^3$ to $5 \times 10^8$ polyhedra depending upon the concentration of the polyhedra suspension from which it was prepared.

C. INITIATION OF INFECTION

Infection was initiated by feeding the larvae polyhedra or by injecting virus particles into the body cavity. Oral infection was accomplished by allowing the larvae to feed on food contaminated with polyhedra. Injections were made with an Agla glass microsyringe (Burroughs-Welcome Co. of England, Fig. 3). The tip of the syringe was fitted with
Fig. 3. Microsyringe and needle apparatus used in injection experiments. 0.5X.
a bored rubber stopper into which a fine glass needle was inserted. Needles were drawn from capillary glass tubing. The distal end of an abdominal proleg was chosen as the point of insertion of the needle because when the needle was withdrawn the muscles contracted and prevented bleeding. Pupae were injected between the antennal folds on the ventral side and adults through the abdominal body wall at the soft intersegmental folds.

D. DIAGNOSIS OF DISEASE

Following the introduction of virus by feeding or injection the insects were reared separately and checked daily. Dead and dying individuals were removed and examined for the presence of disease symptoms under the phase contrast microscope (Leitz-Ortholux). Fresh tissues were simply squashed between the slide and coverslip in a small drop of distilled water. Blood was smeared and allowed to dry before the water and coverslip were applied. Infected nuclei, containing the crystalline polyhedra, were easily recognized (Fig. 4).

Histological methods were used to follow the process of infection in larvae, pupae and adults. Material was fixed in either Bouin's or Gilson's fluids. For pupae and adults the latter was used exclusively because it appeared to soften the cuticle. After fixation specimens were embedded in paraffin (melting point 56° - 58°C Fisher Tissuemat) and sectioned at 4 to 6 microns. Sections were stained with Heidenhain's iron haematoxylin.
Fig. 4. Cells of an infected fifth-instar G. mellonella larva. (a) Live smear preparation of blood cells. Phase-contrast microscope. 700X. (b) Live squash preparation of tissue around a trachea. Phase-contrast microscope. 500X.
An insect is in an advanced stage of infection by the time that polyhedra appear. It was found that a drop of blood or a small piece of fat tissue could be taken from an insect without killing it. These tissues were smeared or squashed on slides and examined under the phase-contrast microscope. If one cell or more in these preparations contained polyhedra the insect was destined to die within one or two days. When polyhedra were found in the blood and fat tissue while the insect was still in the larval stage, infection was so far advanced that death occurred before pupation. The appearance of polyhedra in pupae and adults also indicated advanced infection and imminent death.

E. DESIGN AND ANALYSIS OF INFECTION TESTS

The quantitative response to virus was determined for the wax moth only. Usually four decimal serial dilutions were each tested against 20 or more insects. The concentrations of virus was distributed so that the lowest dilution caused mortality in less than 10% of the test animals and the highest caused more than 90% mortality. In this way the response over the entire range of susceptibility was measured. Insects that died but did not have polyhedra in their tissues were discarded from the tests. The dosage-mortality data were transformed and treated mathematically according to the "Maximum Likelihood Solution" as presented by Finney (1952). Using this method the accuracy of such parameters as median lethal dosages (LD50), response variation, and relative potency were measured.
IV. RESULTS

A. RESPONSE FOLLOWING INFECTION OF FOURTH-INSTAR LARVAE

It has been shown that lepidopterous larvae must ingest relatively large quantities of polyhedra before they become infected but relatively few virus particles injected into the haemocoele will cause infection (Bergold, 1958). This is based on a comparison of median lethal dosages and suggests that many virus particles are inactivated in the alimentary tract or excreted. In the present study the oral and intralymphal dosage-responses were estimated and compared to determine relative effectiveness of each mode of infection over a wide range of dosages as well as at the median lethal dosages (LD50).

1. RESPONSE TO INGESTED VIRUS

A suspension of polyhedra was standardized to $4 \times 10^8$ polyhedra per ml. This stock and three decimal serial dilutions were each tested against 50 fourth-instar larvae. A dose of 0.05 ml was dropped onto a pellet of food and a larva was allowed to consume all of it, after which fresh sterile food was provided. To obtain an estimate of the total mortality-response the insects were reared to the end of the adult stage. The results are shown in Table I.

Mortality increased with dosage, but when the probit transformation was plotted against the logarithm of the dosage the points did not fall.
Table I

Total mortality following ingestion of nuclear polyhedra by fourth-instar G. mellonella larvae.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^4$</td>
<td>42</td>
<td>11</td>
<td>26.2</td>
<td>4.36</td>
</tr>
<tr>
<td>$2 \times 10^5$</td>
<td>47</td>
<td>20</td>
<td>42.6</td>
<td>4.81</td>
</tr>
<tr>
<td>$2 \times 10^6$</td>
<td>42</td>
<td>29</td>
<td>69.0</td>
<td>5.50</td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>46</td>
<td>46</td>
<td>99.5</td>
<td>7.58</td>
</tr>
</tbody>
</table>

Response Formula: Probit Mortality = $(0.784 \times \text{Log Dosage}) + 0.810$

Chi-square Test, $X^2 = 6.988$, Degrees of Freedom = 2, $p = 0.03$

Log Median Lethal Dosage = 5.344

$$LD_{50} = 2.21 \times 10^5 \text{ polyhedra/larva}$$

Heterogeneity Factor = $X^2 = 6.988 \div \frac{D.F.}{2} = 3.494$
on a straight line (Fig. 5). Analysis showed the response was heterogeneous, that is, variation was greater than expected from a normal distribution of errors (Finney, 1952). A heterogeneity factor of 3.494 was calculated and used to adjust parameters estimated from the data.

The logarithm LD50 was 5.344 (LD50 = 2.21 x 10^5 polyhedra per larva). The variance of this estimate was 1.38% and the 95% fiducial limits were 1.891 to 7.331. This means that, based on the data in Table I, any dosage of polyhedra from 7.8 x 10^1 to 2.14 x 10^7 per larva may be expected to cause 50% mortality. Needless to say, this is not a very accurate means of titrating the virus. The fiducial limits, however, may be wide because of excessive variation in the response of only one or two of the three stages of development. Accordingly, the data obtained ten days after the virus was fed were then analysed. At this time the insects had not begun to pupate and all mortality had occurred in the larval stage. This analysis is shown in Table II and Fig. 5. The larval response was homogeneous which indicates that the heterogeneity arose during the pupal and adult stages. The LD50 for larvae was higher (9.84 x 10^5 polyhedra per larva) but its variance was much lower (0.27%) hence the fiducial limits were narrower (5.50 x 10^5 to 1.81 x 10^6). These results show that accurate oral titrations must be based on the larval response only.
Fig. 5. Dosage-response following ingestion of nuclear polyhedra by fourth-instar G. mellonella larvae. The upper line is the combined mortality of larvae, pupae and adults and the lower line is larval mortality only.
Table II

Larval mortality following ingestion of nuclear polyhedra by fourth-instar *G. mellonella* larvae.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^4$</td>
<td>42</td>
<td>4</td>
<td>9.5</td>
<td>3.69</td>
</tr>
<tr>
<td>$2 \times 10^5$</td>
<td>47</td>
<td>11</td>
<td>23.4</td>
<td>4.27</td>
</tr>
<tr>
<td>$2 \times 10^6$</td>
<td>42</td>
<td>25</td>
<td>59.5</td>
<td>5.24</td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>46</td>
<td>41</td>
<td>89.1</td>
<td>6.23</td>
</tr>
</tbody>
</table>

Response Formula: Probit Mortality = $(0.874 \times \text{Log Dosage}) - 0.238$

Chi-square Test, $X^2 = 0.980$, Degrees of Freedom = 2, $p = 0.62$

Log Median Lethal Dosage = 5.993

$$LD_{50} = 9.34 \times 10^5 \text{ polyhedra/larva}$$
2. RESPONSE TO INTRALYMPHAL ADMINISTRATION

The virus particles from $4 \times 10^8$ polyhedra were prepared by alkali treatment and suspended in one ml of distilled water. This stock and three decimal serial dilutions were each tested against 30 fourth-instar larvae. A dose of 0.005 ml was injected into each test larva so that the highest dosage was the virus particles from $2 \times 10^6$ polyhedra per larva. The results are shown in Table III. The percentage mortality increased with dosage. A Chi-square test showed that the response was homogeneous because the data were described by a straight line after the probit-logarithm transformation. The median lethal dosage was the virus from $3.22 \times 10^4$ polyhedra per larva and the 95% fiducial limits of this estimate were $2.02 \times 10^4$ to $5.06 \times 10^4$. This LD50 was much lower and its fiducial limits were narrower than when polyhedra were fed.

This test was repeated using a different polyhedra suspension. The results are shown in Table IV. The LD50 was similar to that obtained in the first test but the slope of the dosage-mortality line appeared to be different. For this reason a Chi-square test was applied to both sets of data and it was found that a common slope described both responses within the limits of error (Table V). The response lines were recalculated using the common slope and there was no significant difference in the median lethal dosages. These results show that a similar response may be expected from intralymphal introduction of different virus preparations.

The insects in these tests were reared to the end of the adult stage in the same manner as those that were fed polyhedra. However, mortality following injection of virus particles occurred only during the larval stage and early part of the pupal stage so that the response was essentially that of larvae.
### Table III

Total mortality following injection of virus particles into fourth-instar *G. mellonella* larvae.

<table>
<thead>
<tr>
<th>Dosage *</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x10³</td>
<td>28</td>
<td>0</td>
<td>0.9</td>
<td>2.63</td>
</tr>
<tr>
<td>2x10⁴</td>
<td>29</td>
<td>10</td>
<td>34.5</td>
<td>4.60</td>
</tr>
<tr>
<td>2x10⁵</td>
<td>26</td>
<td>25</td>
<td>96.2</td>
<td>6.78</td>
</tr>
<tr>
<td>2x10⁶</td>
<td>29</td>
<td>29</td>
<td>99.1</td>
<td>7.38</td>
</tr>
</tbody>
</table>

Response Formula:  
Probit Mortality = (1.820 x Log Dosage) - 3.205

Chi-square Test, $X^2 = 1.13$, Degrees of Freedom = 2, $p = 0.60$

Log Median Lethal Dosage = 4.508

$LD_{50} = 3.22 \times 10^4$ polyhedra/larva

*Dosage expressed as the number of polyhedra dissolved to obtain free virus particles.*
Table IV

Total mortality following injection of virus particles prepared from a different batch of polyhedra from that used to obtain the data in Table III.

<table>
<thead>
<tr>
<th>Dosage*</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8 \times 10^2$</td>
<td>19</td>
<td>0</td>
<td>1.3</td>
<td>2.77</td>
</tr>
<tr>
<td>$8 \times 10^3$</td>
<td>16</td>
<td>4</td>
<td>25.0</td>
<td>4.33</td>
</tr>
<tr>
<td>$8 \times 10^4$</td>
<td>19</td>
<td>12</td>
<td>63.2</td>
<td>5.34</td>
</tr>
<tr>
<td>$8 \times 10^5$</td>
<td>20</td>
<td>20</td>
<td>98.8</td>
<td>7.24</td>
</tr>
</tbody>
</table>

Response Formula: Probit Mortality = \((1.318 \times \text{Log Dosage}) - 0.961\)

Chi-square Test, $x^2 = 0.25$ Degrees of Freedom = 2, $p = 0.90$

Log Median Lethal Dosage = 4.523

\[ LD_{50} = 3.33 \times 10^4 \text{ polyhedra/larva} \]

*Dosage expressed as the number of polyhedra dissolved to obtain free virus particles.
Table V

Analysis of data presented in Tables III and IV

to determine the similarity of response.

<table>
<thead>
<tr>
<th>Test</th>
<th>$SS(x-x)^2$</th>
<th>$SS(x-x)(y-y)$</th>
<th>$SS(y-y)^2$</th>
<th>D. F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.45</td>
<td>28.28</td>
<td>37.63</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>14.24</td>
<td>25.91</td>
<td>48.27</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>35.69</td>
<td>54.19</td>
<td>85.90</td>
<td>4</td>
</tr>
</tbody>
</table>

Common Slope = 1.518

Common Response Formula:

$$\text{Probit Mortality} = (1.518 \times \log \text{Dosage}) - 1.853$$

Chi-square Test, $X^2 = 3.62$, Degrees of Freedom = 4, $p = 0.47$
3. COMPARISON OF RESPONSES

The oral LD50 for larvae was estimated to be $9.84 \times 10^5$ polyhedra while the intralymphal LD50 was the virus particles from $3.22 \times 10^4$ polyhedra. The latter is only 3.3% of the former which means that 96.7% of the consumed virus particles were destroyed, inactivated or excreted before they reached the haemocoel.

Although the LD50's were different the slopes of the dosage-response lines appeared similar indicating that population variation was the same regardless of the method used to introduce the virus (Fig. 6). A Chi-square test, however, showed that the slopes were actually different (Table VI). The slope of the intralymphal response was 1.518 while that of the oral was 0.874. This means that larvae were more variable in response to virus administered by the oral route.

The response lines tend to converge at the lower and diverge at the higher dosages which indicates that as the oral dosage increased a greater proportion of the virus was inactivated (Fig. 6). For example, at log dosage 4.00 about 66% was inactivated before reaching the haemocoel but at log 7.00 almost 99% was destroyed.
Fig. 6. Comparative dosage-response of G. mellonella larvae following ingestion of polyhedra or intralymphal injection of virus particles. Polyhedra fed or particles injected while larvae were in the fourth-instar.
Table VI

Analysis of oral and intralymphal responses showing that a common slope will not describe both.

<table>
<thead>
<tr>
<th>Response</th>
<th>$SS(x-x)^2$</th>
<th>$SS(x-x)(y-y)$</th>
<th>$SS(y-y)^2$</th>
<th>D. F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>75.85</td>
<td>66.27</td>
<td>58.88</td>
<td>2</td>
</tr>
<tr>
<td>Intralymphal</td>
<td>35.69</td>
<td>54.19</td>
<td>85.90</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>111.54</td>
<td>120.46</td>
<td>144.78</td>
<td>6</td>
</tr>
</tbody>
</table>

Chi-square Test, $X^2 = 14.69$, Degrees of Freedom = 6, $p = 0.03$
B. EFFECTS OF METAMORPHOSIS ON INFECTION

1. CHANGES IN RESISTANCE AS LARVAE APPROACH PUPATION

It has been stated that resistance to infection increases with the age of the larva (Bergold, 1958). Conclusive evidence for this view, however, has not yet been published. The following experiments were designed to determine quantitative, as well as qualitative, changes in response as larvae approach pupation.

Newly-molted, fifth-instar, wax moth larvae were each fed $1.8 \times 10^7$ polyhedra and five days later other larvae from the same stock were fed similar doses. Much more mortality occurred when the larvae were fed virus just after molting (Fig. 7). Over 87% of these contracted the disease while only 29% of those fed five days later were infected. The test was repeated with larger doses of virus ($3 \times 10^7$ polyhedra). One group was fed two days after molting into the last instar and another was fed three days later. The results were similar to those obtained in the first experiment (Fig. 7). In a third experiment a sample of 350 larvae was divided into seven equal groups of 50 and each larva was reared individually. Beginning one day before they molted into the last instar and continuing daily, different groups of larvae were fed $1.2 \times 10^7$ polyhedra. The results were the same as those obtained in the preceding experiments (Fig. 7). Total infection decreased from 90% to about 50% as the larvae approached pupation. These three experiments show clearly that as larvae proceed through the fourth and fifth instars they become more resistant to infection.
Fig. 7. Effect of the age at which G. mellonella larvae were fed polyhedra on resistance to disease. Pupation occurred about ten days after larvae molted into the last instar.
To determine the magnitude of this increase in resistance the response of fifth-instar larvae was studied (Table VII) and compared with that of fourth-instar larvae (Table I). Over the whole range of dosage the older larvae were about ten times as resistant as the younger larvae which indicates that all larvae become more resistant as they approach pupation.

Since larvae become more resistant to oral infection they may also become more resistant to intralymphal infection as they approach pupation. Newly-molted, fifth-instar larvae were reared separately. One group was injected with virus immediately while another was not injected until they had started to spin cocoons (seven days later). Four decimal serial dilutions of the same virus preparation were tested against each group. The results are shown in Table VIII. The responses of larvae of both ages were almost identical showing that there is no difference in susceptibility. This result, when compared with that obtained after oral administration of virus, suggests that the factors affecting the increase in resistance as larvae approach pupation are probably located in the alimentary tract.
Table VII

Total mortality following ingestion of nuclear polyhedra by fifth-instar G. mellonella larvae.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x10^4</td>
<td>47</td>
<td>3</td>
<td>6.4</td>
<td>3.48</td>
</tr>
<tr>
<td>2x10^5</td>
<td>41</td>
<td>5</td>
<td>12.2</td>
<td>3.84</td>
</tr>
<tr>
<td>2x10^6</td>
<td>42</td>
<td>16</td>
<td>38.1</td>
<td>4.70</td>
</tr>
<tr>
<td>2x10^7</td>
<td>45</td>
<td>43</td>
<td>95.6</td>
<td>6.71</td>
</tr>
</tbody>
</table>

Response Formula: Probit Mortality = (1.058 x Log Dosage) - 1.607

Chi-square Test, $X^2 = 11.08$, Degrees of Freedom = 2, $p = 0.01$

Log Median Lethal Dosage = 6.245

$LD_{50} = 1.76 \times 10^6$ polyhedra/larva

Heterogeneity Factor = 5.54
Table VIII

Total mortality following injection of virus particles into (A) newly-molted, fifth-instar G. mellonella larvae and (B) larvae beginning to spin cocoons.

<table>
<thead>
<tr>
<th>Dosage*</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x10^2</td>
<td>A 10, B 10</td>
<td>A 0, B 0</td>
<td>2, 2</td>
<td>2.95, 2.95</td>
</tr>
<tr>
<td>5x10^3</td>
<td>A 10, B 10</td>
<td>A 1, B 1</td>
<td>10, 10</td>
<td>3.72, 3.72</td>
</tr>
<tr>
<td>5x10^4</td>
<td>A 10, B 10</td>
<td>A 5, B 6</td>
<td>50, 60</td>
<td>5.00, 5.25</td>
</tr>
<tr>
<td>5x10^5</td>
<td>A 8, B 9</td>
<td>A 7, B 8</td>
<td>88, 89</td>
<td>6.18, 6.23</td>
</tr>
</tbody>
</table>

Response Formulae:

A : Probit Mortality = (1.175 x Log Dosage) - 0.531  
B : Probit Mortality = (1.175 x Log Dosage) - 0.406

LD50's :  
A : 5.09 x 10^4 polyhedra/larva  
B : 3.99 x 10^4 polyhedra/larva

*Dosage expressed as the number of polyhedra dissolved to obtain free virus particles.
2. MORTALITY OF PUPAE AND ADULTS FOLLOWING THE INGESTION OF POLYHEDRA BY LATE LARVAE

In the preceding experiments some of the wax moth larvae that were fed virus during the last instars did not die until they had reached the pupal and adult stages. To determine the proportions that died in each stage, groups of 50 larvae were fed polyhedra at various times before pupation and it was found that as they approached pupation the proportions dying as pupae and adults changed (Fig. 8). When larvae were fed during the early part of the fifth instar most of the mortality occurred before they pupated because the incubation period for the disease was only seven days while the duration of the instar was ten days. On the other hand, if larvae were fed virus during the last part of the fifth instar or three to five days before pupation about 80% of the mortality occurred in the pupal stage. The age at which larvae were fed virus did not affect the proportion that became infected in the adult stage. Infected adults were observed in five of the seven groups. On the whole, about 10% of the insects reached the adult stage and about 40% of these became infected. Diseased adults died within three days of emerging but during this time they often mated and the females laid eggs. It was not determined whether the progeny carried virus but in view of the work on sawflies (Bird, 1961) egg transmission of virus could be expected.

The incubation period of the disease was affected by metamorphosis. When larvae were fed virus a day after molting into the last instar
Fig. 8. Mortality in adult and pupal stages of _G. mellonella_ following ingestion of polyhedra at different stages of larval development.
62% of them became infected and died while still in the larval stage after an incubation period of 7 to 14 days, 14% died in the pupal stage after an incubation period of 13 to 19 days, and 10% died in the adult stage after 22 to 26 days (Fig. 9). It should be noted that the incubation periods for pupae and adults were, respectively, two and three times longer than that for larvae.

Histopathological studies showed that the embryonic regenerative cells are resistant to virus infection and that virus multiplication may be stopped during periods of rapid cellular proliferation, such as, those which occur at pupation and eclosion, thus accounting for the extensions of the incubation period. Last-instar wax moth larvae were each fed $10^7$ polyhedra and sectioned seven days later. Most of them were heavily infected and some had reached the prepupal stage. In all of them the regenerative cells were proliferating and, although the surrounding epithelium was very heavily infected, only early stages of infection were observed (Fig. 10). Similar observations were made on infected silkworm and spruce budworm, *Choristoneura fumiferana* (Clemens), during metamorphosis. When budworm larvae became heavily infected before they reached the prepupal stage cytopathological changes were observed in the peripodium and the base of the wing-disc but the cells of the apex appeared normal (Fig. 11). The wing discs from silkworm prepupae had many more cells undergoing mitosis (Fig. 12) even though the surrounding peripodium was heavily infected. A few of the cells within the disc (1 in 400) contained polyhedra but the majority did not show marked pathological changes (Fig. 13). These results indicate that the embryonic regenerative cells are not susceptible to virus infection until they become fully differentiated.
Fig. 9. Effect of metamorphosis on the rate of mortality following ingestion of polyhedra by fifth-instar *G. mellonella* larvae.
Fig. 10. A section through the wing-bud of a last-instar, *G. mellonella* larva heavily infected with nuclear polyhedrosis virus. Apical cells (a) may be in early stages of infection but infection is more advanced in the posterior cells (b). Polyhedra are present in the peripodium (c) and the epidermis (d). Photomicrograph, 450X.
Fig. 11. A section through the wing-bud of a last-instar *C. fumiferana* larva heavily infected with nuclear polyhedrosis virus. Apical cells (a) appear normal but the posterior cells (b) contain polyhedra. Photomicrograph, 700X.
Fig. 12. A section through the wing-bud of a *B. mori* prepupa heavily infected with nuclear polyhedrosis virus. There are polyhedra in the cells of the peripodium (a) but none are seen in the regenerative cells. Note the cell in metaphase (b). Photomicrograph, 1400X.
Fig. 13. A section of the wing bud of a heavily infected *B. mori* prepupa showing infected cells among the regenerative cells. Photomicrograph, 450X.
3. SUSCEPTIBILITY OF PUPAE AND ADULTS TO
INTRALYMPHAL ADMINISTRATION OF VIRUS PARTICLES

It has been shown that pupae and adults become infected and die from disease if they are fed polyhedra while in the late larval stages. Also, larvae are susceptible to virus if it is injected into the haemocoel. The following tests show that pupae and adults may be infected in the same manner. In one experiment 85 silkworm adults were given intralymphal doses equal to 100 times the larval LD50 (virus from $10^4$ polyhedra) and all of them became heavily infected with nuclear polyhedrosis in $8.5 \pm 1.8$ days. In a second experiment 23 wax moth adults became infected in $8.7 \pm 0.5$ days. These responses are similar to those that could be expected from larvae.

A quantitative estimate of the susceptibility of wax moth pupae relative to larvae was made by injecting serial dilutions of a stock suspension of virus particles into three-day-old pupae and newly molted fifth-instar larvae. The results of this test are shown in Table IX and Fig. 14. Pupae were about twice as susceptible as larvae which indicates that the resistance mechanisms in the pupal haemocoel are not as efficient as are those in the larval haemocoel. The response curves were parallel and both responses were homogeneous showing that all pupae were more susceptible, in other words the increased susceptibility was not confined to only a few individuals.
Table IX

Total mortality following injection of virus particles into newly-molted, fifth-instar G. mellonella larvae and three-day-old pupae.

<table>
<thead>
<tr>
<th>Dosage*</th>
<th>No. in Test</th>
<th>No. Dead</th>
<th>% Mortality</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae Pupae</td>
<td>Larvae Pupae</td>
<td>Larvae Pupae</td>
<td>Larvae</td>
</tr>
<tr>
<td>$5 \times 10^2$</td>
<td>19 10</td>
<td>0 0</td>
<td>1.3 2</td>
<td>2.77</td>
</tr>
<tr>
<td>$5 \times 10^3$</td>
<td>18 13</td>
<td>2 4</td>
<td>17 31</td>
<td>4.05</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>18 12</td>
<td>10 11</td>
<td>78 92</td>
<td>5.77</td>
</tr>
</tbody>
</table>

Response Formulae:

Larvae, Probit Mortality = $(1.678 \times \log \text{Dosage}) - 2.109$

Pupae, Probit Mortality = $(1.678 \times \log \text{Dosage}) - 1.622$

LD50's:

Larvae, $1.73 \times 10^4$ polyhedra/larva

Pupae, $8.83 \times 10^3$ polyhedra/larva

*Dosage expressed as the number of polyhedra dissolved to obtain free virus particles.
Fig. 14. Comparative dosage-response following injection of virus particles into newly-molted, fifth-instar G. mellonella larvae or three-day-old pupae.
C. FATE OF INGESTED VIRUS

The comparison of the oral LD50 with the intralymphal showed that much of the virus ingested is inactivated in the alimentary tract or excreted. This section is concerned with some of the events that occur from the time polyhedra are ingested until the susceptible tissues become infected.

1. DISSOLUTION OF POLYHEDRA

Virus particles are occluded within the polyhedra, the protein matrix of which must be dissolved before the particles can come into contact with susceptible cells. The dissolution very probably takes place in the alimentary tract because when G. mellonella and B. mori larvae were fed food contaminated with polyhedra none were found in the excreted frass. To determine exactly where the polyhedra are dissolved B. mori larvae were fed leaves coated with polyhedra. After several hours of feeding the larvae were killed in hot water and fixed in Gilson's fluid. Sections were stained with iron haematoxylin. Many polyhedra were seen on the surface of the leaf-fragments present in the foregut but soon after the food entered the midgut the polyhedra could not be detected. It was estimated that polyhedra were in the midgut only 20 minutes before they were completely dissolved. B. mori
larvae were used in this experiment because the polyhedra are large (5μ) and their course could be followed through the alimentary tract by observation of normal sections under the light microscope.

2. EXCRETION OF VIRUS PARTICLES

Since polyhedra are dissolved in the midgut the virus particles must be released into the midgut fluid and it is possible that some of the virus particles are excreted in the frass. To determine how much virus is excreted a pellet of food that had been contaminated with 2x10^7 polyhedra was fed to each of ten larvae. They were allowed to feed for two consecutive periods of 16 and 24 hours, respectively, and at the end of each of these periods the frass was collected, macerated and washed twice in 2 ml of distilled water by centrifugation at 4000g. After the second washing the supernatants, in which any virus particles would remain suspended, were combined and centrifuged at 12,000g for one hour. This supernatant was discarded and the pellet was suspended in one ml of water. To test for infectivity 0.002 ml doses of this suspension were injected into each of 20 larvae. As a control, an equal amount of contaminated food was treated in the same way as the frass and tested for infectivity. In an effort to find intact virus particles the remaining portions of the suspensions were centrifuged again at 12,000g and the contents of the pellets were examined in the electron microscope.

No larvae died from virus when injected with suspensions prepared from frass collected at the end of 16 hours but one larva died from disease
when injected with the suspension prepared from the frass excreted during the 16 to 40 hour period. None of the control larvae died from disease. About half of the food was consumed during each period so that 2x10^8 polyhedra were eaten by the 10 larvae. If none of the virus had been inactivated during passage through the alimentary tract, each of the larvae used to test infectivity would have received the virus particles from 2x10^5 polyhedra and a maximum mortality of 88% could have been expected. However, only one larva in 40 (2.5%) died and it therefore appears that most of the virus was inactivated in the alimentary tract. The dosage of virus particles required to cause 2.5% mortality is that contained in about 1000 polyhedra and a comparison of the amount of virus consumed with that excreted shows that 99.5% was inactivated. No particles were observed under the electron microscope.

This test was repeated with some modifications. Twenty larvae were fed contaminated food for a period of 24 hours only. The dosage was increased to 4x10^7 polyhedra per food pellet and about 50% of each pellet was consumed in the time interval. After the frass was collected, macerated and washed and the supernatants concentrated, the final suspension could have contained the virus particles from a maximum of 4x10^8 polyhedra. Each of 40 test larvae was injected with a 0.005 ml dose and could have received the virus particles from about 2x10^6 polyhedra. In this case a maximum mortality of 99.7% could have been expected. None of the larvae died from virus disease which indicated that little or no active virus was excreted.
3. INACTIVATION OF VIRUS PARTICLES BY

THE MIDGUT FLUID

The preceding experiments showed that most of the ingested virus was in some way inactivated in the gut and this led to tests of the in vitro effects of gut fluid. One-tenth ml of midgut fluid was collected from 20 fifth-instar wax moth larvae and diluted with an equal volume of 0.2M Tris buffer at pH 7.2 and held for 30 minutes at 0°C on ice. The virus particles from 5.6x10^7 polyhedra in 0.1 ml of water were added to the buffered gut fluid. This mixture was then incubated at 32°C for 150 minutes. A suspension of virus particles in buffer only was used as a control. After treatment 0.001 ml doses were injected into fifth-instar larvae. The test was repeated using Tris buffers at pH 8.0 and 8.3 to determine the effect of pH over the range that is actually found in the larval midgut (Fig. 17).

Analysis of the infection tests showed that regardless of the pH at which it was incubated the gut fluid completely inactivated the virus while the virus particles suspended in buffer only retained their activity (Table X). When the fluid was extracted and held for 30 minutes at room temperature it lost its activity towards virus particles. This suggests that some kind of enzyme autolysis occurred at room temperature during which the active substance in the fluid was destroyed.
Table X
Inactivation of *G. mellonella* virus particles by midgut fluid.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer Only</th>
<th>Buffer + Virus</th>
<th>Buffer + Virus + Gut fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2*</td>
<td>0/19**</td>
<td>18/20</td>
<td>0/7</td>
</tr>
<tr>
<td>8.0</td>
<td>0/19</td>
<td>18/18</td>
<td>0/12</td>
</tr>
<tr>
<td>8.8</td>
<td>0/17</td>
<td>18/20</td>
<td>0/17</td>
</tr>
<tr>
<td>Total</td>
<td>0/55</td>
<td>54/58</td>
<td>0/36</td>
</tr>
</tbody>
</table>

*Fluid diluted 1:2 in 0.2M tris buffer at pH 7.2, 8.0 and 8.8 and 0°C. Virus particles and diluted fluid held at 32°C for 150 minutes.*

**Denominator = Number of larvae in test. Numerator: Number of larvae that died.
4. EFFECTS OF ALKALINE pH ON POLYHEDRA AND VIRUS PARTICLES

The lumen of the midgut is alkaline in most lepidopterous larvae (Wigglesworth, 1950; Roeder, 1953; Heimpel, 1960) and because Bolle (1898) observed that B. mori polyhedra dissolved in weak alkali solutions it has been postulated that the high pH conditions in the gut cause the dissolution of polyhedra in living larvae. Bergold (1947, 1951, 1953) used 0.005M to 0.008M solutions of sodium carbonate to dissolve the polyhedra and recover the infectious virus particles of the silkmoth, nun moth, gypsy moth and spruce budworm. He balanced the amount of buffer with the amount of polyhedra so that the pH at the beginning of dissolution was 11 but decreased to 10 when dilution was complete. According to Heimpel (1960) the pH of the midgut of the silkmoth larvae may reach 10.3 in certain localized areas but it averages about 9.3. Similarly, that of spruce budworm larvae may reach 9.1 but averages 8.3. These values are lower than the pH required to dissolve polyhedra in vitro and it thus seems that pH may not be the only factor causing the dissolution of polyhedra in the midgut of lepidopterous larvae.

The following experiment was carried out with wax moth polyhedra to determine the pH at which the greatest number of infectious virus particles would be released. Glycine NaOH buffer having a range of pH from 8.6 to 10.6 was used at a concentration of 0.001 M (Gomori, in Colowick and Kaplan, 1955). Solutions having pH values of 9.0, 9.4, 9.8 and 10.4
were tested. Four one-ml quantities of a polyhedral suspension containing $4 \times 10^8$ polyhedra per ml were centrifuged at 3000g for 15 minutes. The supernatants were discarded and each pellet was suspended in 5 ml of buffer for 45 minutes at 25°C. They were then recentrifuged at 3000g to sediment the undissolved polyhedra and leave the released virus particles suspended. The material in the pellets was examined under the electron microscope to observe morphological changes and the supernatants were injected into larvae to determine virus activity. Four decimal serial dilutions were each tested against ten larvae. The response curves were drawn and the median lethal dosages were calculated. The amounts of dissolved protein in the supernatants were kindly estimated by Dr. P. Faulkner, Insect Pathology Research Institute, Sault Ste. Marie. Analyses of the results are shown in Figs. 15 and 16.

There was some dissolution of polyhedra at pH 9.0 but infectious virus was not released (Fig. 15). More protein was dissolved at higher pH values and infectious virus was released. The highest degree of infectivity was obtained from suspensions treated at pH 9.3 and 10.4 and it is significant that they were as infectious as those obtained routinely after sodium carbonate treatment. Observations made under the electron microscope provide additional support. After treatment at pH 9.0 the polyhedra appeared normal (Fig. 16a) but at pH 9.3 and 10.4 they had completely dissolved while the particles freed from the bundles remained suspended in the supernatant fluid.

The foregoing results show that the 0.001M buffer concentration is most effective around pH 10. Ionic strength, however, is known to affect the dissolution of proteins (Haurowitz, 1950) and its effect on wax moth polyhedra was determined by using different concentrations of Glycine-
Fig. 15. Effect of pH on the dissolution of *G. mellonella* polyhedral protein and the release of infectious virus particles.
Fig. 16. Electron micrographs of *G. mellonella* polyhedra and virus particles (Courtesy of Dr. F.T. Bird). (a) Polyhedra after 45 minutes at pH 9.0. 7500X. (b) Thin section of an infected cell showing the bundles of particles within the polyhedra. 45,000X. (c) Bundles of virus particles released at pH 10.4 and centrifuged at 4000g. 20,000X.
NaOH buffers. Molar concentrations of 0.2, 0.02, 0.002 and 0.0002 were tested at various pH levels. Two ml of each buffer solution was used to treat $7 \times 10^7$ polyhedra four hours at 25°C. The number of polyhedra dissolved was estimated by counting the suspensions before and after treatment. The results are shown in Table XI.

Polyhedra were dissolved most effectively at medial ionic strengths. At molar concentration 0.02 and pH 9.7 about 87% of the polyhedra were dissolved but at 0.2M and 0.002M none was dissolved. Similarly, at pH 10.2 the 0.02M concentration was most effective but many polyhedra were also dissolved at 0.2M and 0.002M. The polyhedra were not dissolved at pH 9.1. These results show that ionic strength affects the dissolution of polyhedra only at high pH.

5. pH OF MIDGUT FLUID

The foregoing results show that polyhedra will dissolve in the midgut of wax moth larvae if the pH of the fluid approaches 9.3. Therefore, the pH of the midgut was determined. Individual fourth and fifth-instar larvae were dissected and the digestive fluid was placed in a single-drop calomel electrode fitted to a model G Beckman pH meter. The pH was read within 30 seconds. The mean pH in 69 larvae was 8.04±0.52 (Fig. 17). The distribution was slightly skewed and the range of values was from 7.3 to 9.3. The highest value obtained is far below that necessary to dissolve polyhedra in vitro, thus it appears that dissolution does not depend on pH alone, unless there are regions of high pH in the gut.
Table XI

Effects of pH and ionic strength of glycine-NaOH buffers on *G. mellonella* nuclear polyhedra.

<table>
<thead>
<tr>
<th>Molar Concentration</th>
<th>% of Polyhedra Dissolved ( )</th>
<th>pH of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>.2*</td>
<td>0(9.2)</td>
<td>0(9.8)</td>
</tr>
<tr>
<td>.02</td>
<td>0(9.1)</td>
<td>87(9.7)</td>
</tr>
<tr>
<td>.002</td>
<td>0(9.1)</td>
<td>0(9.7)</td>
</tr>
<tr>
<td>.0002</td>
<td>0(8.6)</td>
<td>0(9.1)</td>
</tr>
</tbody>
</table>

*Polyhedra treated four hours at 24°C.*
Fig. 17. pH of the midgut fluid of fourth and fifth-instar G. mellonella larvae.
6. POSSIBLE PHAGOCYTOSIS OF VIRUS PARTICLES

In mammals the reticulo-endothelial system contains the main phagocytic cells (Wilson and Miles, 1946) but in insects the blood cells and pericardial cells are the principal phagocytes (Wigglesworth, 1950). In mammals, the phagocytes do not appear to exert much influence on the course of virus infections (Burnet, 1960; Smorodintsev, 1960) and in insects the role they play in virus infections has not been investigated. Since phagocytic cells will engulf foreign particles it has been shown that they can be overwhelmed or rendered incompetent by the use of India ink or other finely dispersed particles (Cameron, 1952). In such experiments they are said to be blocked or blockaded. In the present studies attempts were made to block the phagocytes of wax moth larvae by injecting India ink and trypan blue suspensions into the haemocoele.

a. Reaction to India Ink and Trypan Blue

The phagocytic activity of healthy wax moth larvae towards trypan blue and India ink was first investigated. One μl quantities of the ink suspension (1:1, water: stock ink) and 1, 2 and 3 μl doses of 1% trypan blue suspension were injected into the body cavity of fifth-instar larvae. Two hours later small drops of blood were extracted from each and examined using the phase contrast microscope. The relative number of cells containing particles was estimated. After 20 hours more blood was examined and some of the larvae were dissected.
Examination of the blood from larvae injected with ink revealed that after two hours about 70% of the cells contained carbon particles and some cells containing much carbon were clumping to form cysts of 10 to 50 cells. After 20 hours numerous dark concentrations of carbon were observed through the cuticle of the living larvae (Fig. 1a) and an examination of the haemolymph showed that 85% of the blood cells had taken up the carbon particles. During this time none of the ink was excreted, no frass pellets were dropped and larvae did not feed. When larvae were dissected numerous cysts about 0.2 mm in diameter were found floating freely in the lymph. The cysts were observed to circulate through the heart but most of them were concentrated at the posterior end of the body, probably because they had become too large to pass through the heart. Larvae examined three days after they had been injected, were feeding and behaving as normal insects and, although frass was being excreted, the pellets did not contain carbon particles. The cysts of carbon particles remained in the body cavity permanently.

Examination of blood from those larvae which were injected with trypan blue showed that after two hours some of the dye particles were phagocytized but there was no indication of the formation of cysts. The degree of phagocytosis increased with the dose but at 20 hours fewer haemocytes contained dye particles than at two hours (Table XII). This suggests that the particles had been discharged or that the original phagocytes were not in circulation. Much of the dye was excreted during 20 hours and, although all the frass pellets were dyed blue, the normal number of frass pellets were dropped. The remaining dye became concentrated in the pericardial cells (Fig. 19) where it persisted throughout metamorphosis.
Fig. 18. Distribution of India ink particles in a fifth-instar *G. mellonella* larva 20 hours after the ink was injected. 4X. Photographed by D. C. Anderson.
Table XII
Phagocytosis of trypan blue particles by fifth-instar G. mellonella larvae.

<table>
<thead>
<tr>
<th>Dosage (μl)</th>
<th>No. of Larvae</th>
<th>% of Cells Containing Dye Particles</th>
<th>Number Frass Pellets per Larva 2 hrs.</th>
<th>20 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>24</td>
<td>14</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>26</td>
<td>26</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>75</td>
<td>32</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Fig. 19. Localization of trypan blue in the pericardial cells of a fifth-instar G. mellonella larvae 20 hours after the dye was injected. 3.5x. Photographed by D. C. Anderson.
b. Infection by Virus Particles Following Blockage of the Phagocytes

The effect of blocking the pericardial cells with trypan blue on subsequent infection by virus was determined by injecting virus particles into larvae that had been injected with dye 24 hours previously. This treatment had no effect on the subsequent infection caused by the virus particles (Table XIII). The response of larvae that contained dye was the same as that of those which did not and the LD50's were the virus particles from $2.1 \times 10^4$ and $2.3 \times 10^4$ polyhedra, respectively.

The effect of injection with India ink on subsequent virus infection was determined by injecting virus particles into larvae that had been injected with ink 4, 20 or 48 hours previously. Each larva was injected with one μl of a 1:1 suspension of ink and water and challenged with a one μl dose of virus containing the particles from $1.5 \times 10^4$ polyhedra. As a control an equal number were injected with virus particles only. The same suspension of virus particles was used throughout the experiment. Larvae treated with India ink were more susceptible to virus particles than were the controls (Table XIV). Four hours after receiving the ink their resistance was lowered and remained so through 20 and 48 hours.

The phagocytosis experiments showed that 70% and 85% of the blood cells had phagocytised carbon particles after two hours and 20 hours, respectively and it appears that these cells cannot phagocytise virus particles after they have taken up a large number of ink particles.

The response of fifth-instar larvae was studied over a range of dos-
Table XIII

Effect of blocking the pericardial cells with trypan blue on the susceptibility of *G. mellonella* larvae to injected virus particles.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>No. of Larvae</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dye Only</td>
</tr>
<tr>
<td>1.2x10^4</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.2x10^5</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>1.2x10^6</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Microlitre (µl) doses of dye were injected 24 hours before the virus particles.*
Table XIV

Effect of blocking the phagocytic blood cells with India ink on the susceptibility of *G. mellonella* larvae to injected virus particles.

<table>
<thead>
<tr>
<th>Time Between Administration of Ink and Virus* (hours)</th>
<th>No. of Larvae</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ink and Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus only</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>20</td>
<td>83</td>
</tr>
</tbody>
</table>

*Microlitre (μl) doses of ink were injected before the virus particles. Each larva received the virus particles from \(1.5 \times 10^4\) polyhedra.*
ages of virus particles to determine the quantitative effect of injecting India ink 24 hours previously. As a control similar larvae were injected with distilled water and challenged with the same suspensions of virus particles. As in the previous test, blockage of the phagocytes rendered the larvae more susceptible to virus infection (Fig. 20). The LD50 for the treated larvae was the virus particles from 1.4x10^3 polyhedra while that for the controls was the virus particles from 2.14x10^4 polyhedra. Comparison of these two values shows that at least 93% of the virus particles injected into the haemolymph of larvae may be phagocytised by blood cells. Except for the increased susceptibility, the treated larvae responded in the same manner as the controls. Chi-square tests showed that a common slope described both sets of data indicating that all larvae have about the same proportion of phagocytes which are active against virus particles and which may be blocked by India ink. The efficiency of phagocytosis does not change with dosage but the total number of virus particles inactivated increases with dosage. For example, about 92% of the particles at both LD10 and LD90 are inactivated but when the total number of particles inactivated at LD10 (2.43x10^3) is compared with that at LD90 (1.26x10^5) it is apparent that 52 times as much virus was inactivated at the higher dosage.
Fig. 20. Effects of blocking the phagocytes with India ink on the susceptibility of *G. mellonella* larvae to intralymphal injection of virus particles.
7. VACCINATION WITH LIVE VIRUS

The following experiments were designed to determine whether there is an immune response in wax moth larvae to sublethal doses of live nuclear polyhedrosis virus. The virus particles in 2x10^3 polyhedra was used as a vaccination dosage. Two days later some of these larvae were injected with a challenge dose of particles from 2x10^6 polyhedra and at seven days others were challenged. There was no difference in susceptibility of vaccinated larvae and untreated larvae at either two or seven days following vaccination (Table XV). About 5.3% of the larvae that were vaccinated but not challenged died from virus disease and none of the untreated larvae became infected. These results indicate that a small dose of virus particles injected into the haemolymph does not induce an immune response in wax moth larvae.

Multiple vaccinations usually cause the immune response to be enhanced in mammals. In view of this larvae were vaccinated twice with the virus particles from 10^3 polyhedra. The second vaccination was given 20 hours after the first and the larvae were challenged with lethal doses of virus particles 48 hours after the first vaccination. For a control some larvae were vaccinated only once and others were not vaccinated at all. As in the first experiment an immune response was not observed (Fig. 21). Conversely, the multiple vaccination had a sensitizing effect. The larvae that received two vaccinations were about eight times as susceptible as the controls. This suggests that a number of small doses of virus particles injected into the body cavity are
Table XV

Effects of a single vaccination with live virus particles on the susceptibility of *G. mellonella* larvae to a subsequent lethal dose of virus particles.

<table>
<thead>
<tr>
<th></th>
<th>No. of Larvae</th>
<th>No. Dead</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vaccination Only</em></td>
<td>19</td>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>Challenged;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days after vaccination</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>7 days after vaccination</td>
<td>20</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>not vaccinated</td>
<td>40</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Vaccination dosage = the virus particles from 2x10^3 polyhedra
Challenge dosage = the virus particles from 2x10^6 polyhedra
Fig. 21. Effect of multiple vaccination with live virus particles on the susceptibility of G. mellonella larvae to subsequent lethal doses of virus particles.
able to cause more mortality than a single large dose.
V. DISCUSSION

The results of the studies on host-response to virus suggest that certain precautions must be taken before the oral activity of polyhedra suspensions are compared. Estimates of titer should be based only on mortality occurring in the larval stage because the collective response of larvae, pupae and adults is so heterogeneous that extremely wide confidence limits are obtained. The age at which larvae are fed virus is also important because they become more resistant as they approach pupation. Fifth-instar larvae are more than ten times as resistant as fourth-instar larvae. These limitations do not apply to estimates of intralymphal activity which may be based on the response of fourth- or fifth-instar larvae or pupae. The only difference in these three stages is that pupae are about twice as susceptible as larvae.

The heterogeneity of the collective response of G. mellonella larvae, pupae and adults following oral administration of virus to larvae in the late instars indicates that dosage may not be a very important factor in epizootics. For example, when fourth-instar larvae are fed virus the collective response is heterogeneous and has wide fiducial limits. A dosage of $10^5$ polyhedra per larva may be expected to cause between 16 and 60 per cent mortality and $10^7$ polyhedra per larvae may cause from 65 to 99 per cent mortality. These observations suggest that virus distribution may be the most important factor to
consider when introducing viruses for natural control, that is, lower dosages distributed over a larger area may be most effective because more larvae will come in contact with the virus.

There is a wider range of response to viruses than to insecticides. Bucher (1956) reported that the slopes for both oral and intralymphal responses of silkworm and forest tent caterpillar, *Melocosoma disstria* (Hbn.), to viruses were about unity while those for certain insecticides and *Bacillus thuringiensis* toxin were much higher. In the present study, some wax moth larvae were infected by very small doses while others resisted even the largest quantities. This was particularly true when virus was fed. By extrapolating from the response curves (Fig. 6) it was estimated that if a group of fourth-instar larvae were each fed $3 \times 10^4$ polyhedra about 10% would become infected but, on the other hand, 10% would survive doses of $3 \times 10^7$ polyhedra.

When wax moth larvae were fed virus late in the last instar most of the infection occurred in the pupal stage with small amounts occurring in the larval and adult stages. Pupal infection following oral administration of polyhedra to larvae has been observed previously (Glaser, 1915; Bergold, 1947; Stairs, 1960) but this is the first observation of adult infection among Lepidoptera. Bird (1949, 1953) has shown that adults of *D. hercyniae* (Htg.) may be infected and that they transmit the virus to some of their progeny. It was not determined whether infected wax moth adults transmitted the virus to their progeny but it is possible that they do.

The extensions of the incubation period during metamorphosis suggests that the infection process was delayed. The period from oral administration of virus to mortality for larvae was eight days but for
pupae and adults this period was extended to 16 and 24 days, respectively. The distribution of mortality over the three stages was trimodal, occurring at distinct periods which suggests that virus multiplication was interrupted twice. A similar effect was observed by Bird (1949, 1953) for the sawfly, D. hercyniae, where infection is stopped during metamorphosis because the regenerative cells are believed to be resistant to virus until they become differentiated. The regenerative cells of the wax moth also appear to be resistant (Fig. 10) but instead of one delay, as in the sawfly, there are two delays, one at pupation and the other at eclosion. Preceding each of these periods a great many cells are replaced by regenerative cells, hence new cells become susceptible as they differentiate but essentially this is a new infection and the overall infection process is interrupted. The degree of infection occurring in the larval stage probably affects the period during which the insect will die. If a larva is moderately infected before pupation it will probably die during the pupal stage but if it is only lightly infected it may survive the pupal stage and die in the adult stage.

A number of studies have shown that viruses inhibit mitosis. According to Marcus and Puck (1958) a single Newcastle disease virus particle successfully penetrating a Hela cell prevents that cell from undergoing mitosis. Herpes virus has a similar effect on Hela cells where mitosis is inhibited in less than an hour after virus adsorption and virus particles that absorb but do not infect fail to inhibit mitosis (Stoker and Newton, 1958). Histological evidence shows that the embryonic regenerative cells in sawflies (Bird, 1949, 1953) and Lepidoptera continue to multiply even after all adjacent functional
cells have been infected which, in turn, indicates that the embryonic cells are highly resistant if not completely immune to virus attack.

Bergold (1947) suggested that polyhedra are dissolved in the midgut of lepidopterous larvae but this was demonstrated for the first time in the present study where polyhedra were observed to disappear in the anterior part of the midgut of B. mori larvae. It has been assumed that the pH of the gut fluid is the chief factor responsible for the dissolution of the polyhedra because in most Lepidoptera this fluid is alkaline (Heimpel, 1960) and because polyhedra are dissolved in vitro by weak alkaline solutions. In general, this assumption was not confirmed in the present study for the pH of the larval midgut of G. mellonella was found to be too low to dissolve polyhedra. Regional differences in pH were not measured, however, and it is still possible that the pH at some part of the midgut is high enough to cause polyhedra to dissolve. More probably, there is a proteinase present that attacks the polyhedral crystals and the action of this proteinase may be enhanced by reducing agents. In the presence of 0.02M to 0.125M cysteine, which breaks the -5-5- bonds, the proteinase of clothes moth larvae, Tineola bisselliella L., digests wool more effectively (Powning and Irzykiewicz, 1962).

Most of the virus that is ingested by wax moth larvae is inactivated in the alimentary tract. It was found that alkaline buffers having a range from 7.0 to 10.4 did not inactivate free virus particles but the midgut fluid inactivated virus very efficiently when diluted 1:2 in Tris buffer at pH 8.0. The activity of the fluid decreased rapidly at room temperature but remained fairly constant at 0°C. Aizawa (1962)
reported a similar activity for the gut fluid from silkworm larvae and suggested that the active principle is an enzyme since it is heat labile and may be extracted by methyl alcohol. Regardless of the nature of these inactivating factors, it appears that they are an important means of defense against virus infections.

The efficiency with which virus is inactivated in the alimentary tract increases directly with the number of polyhedra ingested. At log dosage 4.00 about 34% reaches the haemocoel in an infective condition whereas at log dosage 7.00 only 1.0% reaches the body cavity. This suggests that low or medial dosages may be used more economically than high dosages to obtain control of the host provided that an extremely high percentage kill is not essential. In other words, the high dosages of virus that would be used to produce high mortality might be more efficiently disseminated at lower dosages over a much wider area.

The phagocytic blood cells may be able to remove virus particles from the haemolymph and inactivate them. This conclusion is based on the fact that wax moth larvae become more susceptible to intralymphal infection after their phagocytes have been blocked by India ink particles. The pericardial cells do not appear to phagocytise virus particles because blocking them with trypan blue particles did not affect larval susceptibility to virus. There is some evidence which suggests that blocking the phagocytes may affect other systems. According to Wigglesworth (1959) blockade of the phagocytes of Rhodnius with India ink prevented the secretion of the growth and molting hormone and the nymphal stage was extended. In the wax moth pupation occurred normally after treatment with India ink, however, larvae neither fed nor
excreted frass for 24 hours after being injected with India ink. This indicates that the ink had side effects on the physiology of the larvae. Nevertheless, two days after they had been treated with ink they were behaving as normal insects and their susceptibility to virus particles was still higher than untreated larvae so it seems that the blockage of phagocytes really increased susceptibility.

Wax moth larvae that were vaccinated with small doses of virus particles did not develop immunity to subsequent lethal doses. This suggests that a humoral immune reaction does not occur and that no antibody is produced in response to vaccination with living virus particles. This result parallels that obtained by Briggs (1958) who could not detect immunity or antibodies in a number of Lepidopterous larvae vaccinated with heat-killed, bacterial vaccines. Similarly, Stephens (1959) was not able to detect antibodies like those found in mammals but succeeded in demonstrating an immune response of wax moth larvae to heat-killed vaccines of the bacterium, *Pseudomonas aeruginosa*. This response appears rapidly, reaches a peak in 24 hours and decreases slowly thereafter until it disappears in about four days. The immune principle is located in the protein fraction of the blood serum but it has not been identified (Stephens and Marshall, 1962). Tests with inactivated virus particles were not made but in view of the absence of an immune response to living particles it is unlikely that one will be induced by such a vaccine. Inactivated virus, however, may interfere with infection by living virus as it does in many cases of vertebrate infections (Burnet, 1960).
VI. SUMMARY

The wax moth, *Galleria mellonella* L, and its specific nuclear polyhedrosis virus was used as a model test system to obtain basic information regarding virus-host relationships in Lepidoptera.

The dosage-mortality response following ingestion of polyhedra was different from that following injection of virus particles into the haemocoele. When fourth- or fifth-instar larvae were fed polyhedra mortality occurred in the larvae, pupal and adult stages and the combined response was heterogeneous. The larval response was homogeneous, however, indicating that the most accurate titrations will be obtained by using this response only. When fifth-instar larvae were injected with virus particles mortality occurred in the larval stage and early part of the pupal stage; no late pupae or adults died. The response was homogeneous and the LD50 was lower than that obtained following oral ingestion of polyhedra which shows that larvae are less variable and much more susceptible to injected virus particles. Quantitative comparison of the LD50's shows that only 3.3% of the virus particles in the ingested polyhedra could have reached the haemocoele. Furthermore, the relative efficiency of inactivation increased directly with dosage.

Resistance to oral infection increased with the age of the larvae. Fifth-instar, *G. mellonella* larvae were about ten times as resistant as
fourth-instar larvae. Resistance to intralymphal infection did not change with larval age which suggests that the factors affecting the increase in resistance to oral infection are probably located in the alimentary tract.

Following ingestion of polyhedra by fourth- or fifth-instar, wax moth larvae mortality from virus disease occurred in the larval, pupal and adult stages. The proportions dying in the larval and pupal stages varied with the age at which larvae were fed polyhedra but about three per cent died in the adult stage regardless of when they were fed.

The incubation period of the disease was extended by metamorphosis. The incubation periods for pupae and adults, respectively, were two and three times longer than that for larvae. Histopathological studies showed that the embryonic regenerative cells are resistant to virus infection until they become fully differentiated, thus, the infection process is interrupted during the periods of rapid cellular proliferation just before pupation and eclosion. During these periods many new cells are produced that must be infected before the insect can be killed by the virus. Similar observations were made on Bombyx mori and Choristoneura fumiferana (Clemens).

Adults and pupae of G. mellonella and B. mori were susceptible to virus particles injected into the haemocoel. Three-day-old pupae were about twice as susceptible as fifth-instar larvae.

The fate of polyhedra and virus particles was traced. Histological sections of B. mori larvae revealed that the polyhedra are dissolved soon after they enter the midgut. Significant amounts of infectious virus was not excreted by G. mellonella larvae feeding on contaminated food. It was found that midgut fluid inactivated virus particles in vitro
very effectively. The antiviral substances are probably enzymes because the fluid did not retain its activity at room temperature and because the pH conditions in the midgut were too low to inactivate virus particles or dissolve polyhedra.

Larvae whose phagocytes were blockaded by India ink were rendered less resistant to virus infection. Resistance decreased within four hours and remained low for at least 48 hours following the ink treatment. Quantitative tests indicated that about 93% of the virus particles reaching the haemocoel may be phagocytised. Blockading the pericardial cells with trypan blue did not affect larval resistance which suggests that these cells are not concerned with phagocytosis of virus particles.

Vaccination with sublethal doses of living virus particles did not induce an immune response in G. mellonella larvae. On the contrary, larvae that received two vaccinations became more susceptible to lethal dosages.
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