A VIRUS DISEASE OF LEATHERJACKETS

John B. Carter

The West of Scotland Agricultural College,
Bacteriology Department,
Auchincruive,
Ayr.

Submitted for the degree of
Ph.D. in the University of
Glasgow.

May, 1971.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

A *Tipula* iridescent virus (TIV) infection in the leatherjacket, *Tipula oleracea* (Linnaeus), was investigated in an attempt to assess whether TIV might be suitable as an agent for the biological control of leatherjackets. These insects are agricultural pests, and at present are controlled by chemicals which often affect many harmless and beneficial organisms. The investigations were mainly concerned with the mode of transmission of the virus and with effects of the environment upon the disease in the insect.

Techniques for the assay of TIV were devised. A response-time method, which involves measuring incubation periods in TIV-injected larvae, did not give satisfactory estimates of TIV titres unless large numbers of larvae were used. There appears to be wide variability in the susceptibility of larvae to infection with TIV, which also limits the precision of TIV estimates by the usual LD50 method. Antibody-sensitised latex particles were used to detect and assay large concentrations of TIV.

All stages in the life-cycle of *T. oleracea*, except the embryo, were found to be susceptible to TIV. Iridescence was observed in infected larvae in all four instars, in pupae, and in adults of both sexes.

The transmission of TIV from infected to non-infected leatherjackets was demonstrated. It is suggested that TIV is transmitted mainly by non-infected larvae feeding upon the cadavers of infected larvae, and that a new generation becomes infected by feeding upon the cadavers of infected fourth-instar larvae from the previous generation.

TIV-infected larvae were shown to excrete the virus, but not in quantities which are likely to serve as a source of infection, as leatherjackets only become infected *per os* if a large dose of virus is ingested. Inactivation of the virus by the gut fluids was considered as a possible reason for its low infectivity when ingested by the larvae, but the infectivity of a TIV suspension was found to be unaffected by larval gut fluids *in vitro*.

It was shown that the rate of TIV replication is markedly affected by temperature, and it is suggested that the disease develops most rapidly during the warmest months of the year, with a very slow rate of development in winter.

TIV was found to be a fairly stable virus, the infectivities of virus suspensions at 20°, 5° and -16° gradually declining over a period of one year.

It is concluded that investigations into the control of leatherjackets with TIV merit support, although it is possible that the initiation of TIV epizootics may prove to be a difficult task. Some of the problems which might be encountered in a programme of biological control are discussed.

GENERAL INTRODUCTION

A. LITERATURE REVIEW

Tipula iridescent virus

TIV was first reported by Xeros (1954) in the leatherjacket, Tipula paludosa (Meigen). It was so named because in later stages of the disease the infected fat body appears iridescent purple, blue and/or green in reflected light, and orange in transmitted light. A pellet of the virus is similarly iridescent (Williams and Smith, 1957); differences in colour have been ascribed to the degree of hydration of the pellet (Williams and Smith, 1957) and the species of host insect in which the virus was grown (Smith and Hills, 1959). TIV-infected leatherjackets have been found in England (Xeros, 1954 and 1956), Scotland (Dunn, 1966) and France (Vago, 1968).

TIV has been studied in a number of laboratories in recent years, and many of its properties are now known. An unusual property of this virus is that it appears to have a very wide host range. In addition to T. paludosa larvae, it has been found in T. pagana (Meigen), T. marmorata (Meigen) and T. hortulana (Meigen) (Stewart, 1969), and it is probable that many other tipulid species are infected in nature. T. oleracea (Linnaeus), T. livida (Van der Wulp) and other unidentified species of Tipula have been infected with TIV in the laboratory (Smith and Rivers, 1959). The virus has also been shown to be infective for other members of the Diptera, e.g. the larva of the bluebottle fly, Calliphora vomitoria (Linnaeus), and also for members of other orders, e.g. the larva of the cabbage white butterfly, Pieris brassicae (Linnaeus) (Lepidoptera) and the larva of the mealworm, Tenebrio molitor (Linnaeus) (Coleoptera). Cell cultures of the emperor

gum moth, Antheraea eucalypti (Scott), have also been infected with TIV (Hukuhara and Hashimoto, 1967).

Bailey, Gibbs and Woods (1970) gave the virus cryptogram (Gibbs et al, 1966) for TIV as D/2 : 126/15 : S/S : I/* (see footnote), although various workers have given different values for the percentage of DNA in the virion. Smith, J.D. (unpublished, quoted by Smith, K.M., 1958b) gave a figure of about 15% DNA, but values of 12.4% (Thomas, 1961), 16% (Allison and Burke, 1962), 17% (Glitz, Hills and Rivers, 1968) and 19% (Kalmakoff and Tremaine, 1968) have also been obtained.

There are also conflicting reports concerning the diameter of the virion. These are probably due, at least in part, to different states of the virions during measurement. Arndt and Beeman (unpublished, quoted by Klug, Franklin and Humphreys - Owen, 1959) estimated the diameter of TIV particles in suspension to be 180 nm, whereas Thomas (1961) found the diameter of the freeze-dried particle to be 130 nm. Williams and Smith (1957) also obtained a value of 130 nm by measuring the smallest centre-to-centre distance of two particles. Xeros (1954) quoted a figure of 100 nm, and Hills and Smith (1959) one of 144 nm. There is no doubt, however, that the TIV particle is large compared with most other viruses.

The shape of the particle was shown to be an icosahedron by Williams and Smith (1958), who used a double-shadowing technique in the electron microscope. The number of protein subunits in the particle was estimated

---

Explanation of cryptogram

D	=	DNA	S	=	essentially spherical particle
2	=	double-stranded	S	=	essentially spherical nucleocapsid
126	=	molecular weight of DNA (in millions)	I	=	insect hosts
15	=	percentage DNA	*	=	no vectors known

to be 812 by Smith and Hills (1962), but Wrigley (1970) has suggested that the number is probably 1,472. The amino acid composition of TIV protein has been studied by Kawase and Hukuhara (1967) and Kalamakoff and Tremaine (unpublished, quoted by Tremaine and Goldsack, 1968).

Glitz et al (1968) found the LD63 (the dose infecting 63% of the test insects) of TIV to be 2.5×10^3 particles, by injection, for T. paludosa, and 5.0×10^3 particles for T. oleracea. These workers did not state their reasons for using the LD63 end-point. Much higher doses are required to produce infection when the virus is introduced per os (Rivers, 1966a).

The events inside the TIV-infected cell have been investigated by a number of workers. Replication takes place primarily in the larval fat body (Xeros, 1954), the cells at the periphery being infected first (Kanyuka and Pronina, 1970). Other tissues, e.g. muscle and hypodermis (Xeros, 1956), become infected later. Virions enter the cells by phagocytosis (Xeros, 1964a), and there is some evidence that they are taken into lysosomes (Younghusband and Lee, 1970). Replication occurs in the cytoplasm, which enlarges enormously (Xeros, 1956). Anderson, Armstrong and Niven (1959) reported an increase in nuclear size, while Norris (1970), studying the disease in larvae of the greater wax moth, Galleria mellonella (Linnaeus), observed a slight increase in size of the nucleus initially, but later it began to shrink. Oliveira and Ponsen (1966), using the fluorescent antibody technique, studied the production of TIV antigen in haemocytes from P. brassicae larvae inoculated with TIV.

Using fluorescence microscopy, the cytoplasm of infected cells was found to emit strong fluorescence of the DNA type by Armstrong and Niven

(1957), who also reported that no obvious changes occur in the nuclei of infected cells, except enlargement of the nucleolus, which fluoresces bright red. Kanyuka (1968), studying TIV-infected larvae of G. mellonella and the gypsy moth, Porthetria dispar (Linnaeus), reported an increase in nuclear DNA and RNA. Morris (1970), however, using autoradiography, found only a slight increase in DNA synthesis and no appreciable increase in RNA synthesis in infected nuclei of G. mellonella, but he found an increase in RNA synthesis in the cytoplasm, especially in areas of virus multiplication. Thus, it appears that most of the events concerned with the replication of TIV take place in the cytoplasm.

The electron microscope has been used to study the formation of virions in the infected cell (Smith, 1958a and 1958b; Bird, 1961a and 1962; Younghusband and Lee, 1969). Many empty protein coats were observed by Smith and Hills (1959), especially in the early stages of the disease; each virion can be seen to be surrounded by two membranes. The endoplasmic reticulum gradually disappears, and it is thought that the empty coats become filled with DNA (Smith 1958b). These cells disintegrate, liberating their contents into the blood, and the larva becomes purplish-white in colour (Xeros, 1954). Williams and Smith (1957) estimated that virus accounts for approximately 25% dry weight of a diseased larva. Death generally occurs 2 - 4 weeks after iridescence has appeared (Xeros, 1954).

Smith, Hills and Rivers (1961) inoculated TIV into larvae of many insect species, and in no case were any latent virus infections stimulated into activity. Kanyuka (1965), however, reported that injection of TIV into silkworm, Bombyx mori (Linnaeus), larvae and G. mellonella larvae

resulted in the activation of nuclear polyhedrosis viruses (NPVs) specific for each of these species.

Since the discovery of TIV, several similar viruses have been found in other insects. These have been reviewed by Smith (1967), Vago (1968) and Bellett (1968). Iridescent viruses have been found in larvae of 1) the pruinose scarab, Sericesthis pruinosa (Dalman) (Steinhaus and Leutenegger, 1963); 2) the mosquitoes Aedes taeniorhynchus (Wiedemann) (Clark, Kellen and Lum, 1965), Aedes annulipes (Meigen) and Aedes cantans (Meigen) (Weiser, 1965), Aedes stimulans (Walker) (Anderson, 1970) and again in A. cantans (T.W. Tinsley and J.S. Robertson, unpublished, quoted in Tinsley and Kelly, 1970); 3) the rice stem borer, Chilo suppressalis (Walker) (Fukaya and Hasu, 1966); 4) the blackfly, Simulium ornatum (Meigen) (Weiser, 1968); 5) a biting midge (Culicoides sp.) (Chapman et al, 1968); 6) Wiseana cervinata (Kalamakoff and Robertson, 1970); and Witlesia sabulosella (J.S. Robertson and M. Fowler, unpublished, quoted in Tinsley and Kelly, 1970). Stoltz, Hilsenhoff and Stich (1968) described a virus resembling the iridescent viruses from larvae of the midge, Chironomus plumosus (Linnaeus), but no iridescence was observed in the infected insects.

The insect hosts of these viruses are only distantly related phylogenetically, and the relationships of the iridescent viruses with each other are somewhat problematical. TIV and Sericesthis iridescent virus (SIV) have been shown to be related serologically (Day and Mercer, 1964; Cunningham and Tinsley, 1968), although there are serological differences between them (Bellett and Mercer, 1964; Glitz et al, 1968).

TIV and SIV were found to be unrelated to the mosquito iridescent virus (MIV) from A. taeniorhynchus when compared by the complement fixation test (Cunningham and Tinsley, 1968). Chilo iridescent virus (CIV) was reported to be serologically unrelated to TIV (Fukaya and Hasu, personal communication to Tojo and Kodama, 1968) or SIV (Day, personal communication to Tojo and Kodama, 1968). CIV and TIV are distinguishable in sedimentation rate and chromatographic behaviour (Tojo and Kodama, 1968). Hatta (1970) found the ultra-violet absorption spectrum and the amino acid composition of the MIV from A. taeniorhynchus and TIV to be very similar, but the diameter and sedimentation coefficient of the MIV were both larger than those of TIV and SIV.

The base ratios and molecular weights of the nucleic acids of TIV, SIV and CIV were found to be similar by Bellett and Inman (1967), and this led Bellett (1967) to suggest that the iridescent viruses are homologous, i.e. that their nucleic acids have common sequences. Evidence of base-sequence homology between the nucleic acids of TIV, SIV and CIV was obtained in hybridisation experiments (Bellett and Fenner, 1968). It may well be that the iridescent viruses are unrelated phylogenetically, but are an example of convergent evolution. Tinsley and Kelly (1970) have suggested that, until the relationships between the iridescent viruses are better understood, each should be known by a number, so that alterations and additions to the group can be easily made. In their system TIV is designated as iridescent virus Type 1.

The leatherjacket (Tipula) and its control

There are 59 British species of Tipula (Freeman, 1967) and 291 British species of Tipulidae (crane-flies) (Burton, 1968). T. paludosa is very common in the United Kingdom, especially in the North and West, and also all over Northern Europe (Edwards and Heath, 1964); it is also found in North America (Wilkinson and MacCarthy, 1967). T. oleracea is much less common than T. paludosa.

Leatherjackets, also known as 'bots' (Thomson, 1926), 'tory worms', 'the grub' (Rennie, 1927) and 'cutworms' (Willis, 1963) are the larval stages of crane-flies. The life-histories of T. paludosa and T. oleracea have been studied by a number of workers. T. paludosa is univoltine with adults on the wing from July to September (Ahmed, 1968), whereas T. oleracea is usually bivoltine (Laughlin, 1960), with adults on the wing in May, and again in August (Freeman, 1967). Rennie (1927) observed that "polygamy is notoriously rife" among T. paludosa adults.

The eggs are laid in the soil, and when the larvae hatch they grow very fast in the autumn, slowly through the winter, and moderately fast in the spring and early summer (Laughlin, 1967). They commence feeding, both above and below ground, shortly after dark, and continue until near dawn (Ahmed, 1968).

Leatherjackets have long been recognised as agricultural pests. Curtis (1849) reported them feeding upon turnips, beet, carrots, cabbages and potatoes, although later investigators, e.g. White (1968), state that leatherjackets do not damage potatoes. They are also known as pests of grassland, cereals, peas, beans, and seedling forest crops (MacDougall, 1932)

Some crops may be damaged before the hypocotyl emerges above ground-level (Long, 1969).

Grennan (1966) found that small populations of leatherjackets cause much unnoticed damage to pasture; yields were much lower on leatherjacket-damaged plots. White (1968) stated that when the sward starts dying off due to leatherjacket damage, 4.9 - 6.2 million larvae per hectare are usually present. He estimated that a leatherjacket population of 4.4 million per hectare would result in a loss of £11.85 per hectare. He has also estimated (White, 1966) that the average annual value of cereal losses in Britain due to leatherjacket damage may total £300,000 - £600,000.

Leatherjackets, therefore, can cause quite considerable losses to British agriculture. At the present time they are controlled by chemical insecticides, with DDT and benzene hexachloride being widely used (British Agrochemicals Association, 1970). Other substances which have been used are lead arsenate (Dawson and Ferro, 1936) ortho-dichlorobenzene (Dawson, 1932), gammexane (Escritt, 1947), and aldrin and parathion (White, 1967).

There is now much concern about the presence of many of these substances in the environment. Carson (1962) was one of the first to voice this concern, and she obtained wide recognition for her views, even if she did state them in a somewhat sensational manner. Newson (1967) pointed out some of the effects of insecticides: 1) predators and parasites of the pests are killed, as well as the pests themselves; 2) there is a considerable hazard to birds and fishes which feed on insects which contain insecticides in their tissues; 3) chlorinated hydrocarbons are

exceptionally toxic to fish; 4) birds are affected by the spraying of insecticides. Some of the risks attendant in the use of insecticides against leatherjackets have been voiced. White (1963) mentioned the risks to wild life, and the possible deleterious long-term effects of the accumulation of pesticides in the soil. Willis (1963) gave a warning about the residue-risk of using DDT for leatherjacket control, and stated that the insecticide should not be applied to pasture until grazing has ceased. Willis (1965) also warned that spraying with DDT to control leatherjackets may cause serious damage to certain varieties of barley.

DDE, a common metabolite of DDT, has been shown to cause egg-shell thinning in a number of species of birds (e.g. see Wiemeyer and Porter, 1970). Jensen et al (1969) reported the presence of DDT and para-dichlorobenzene in marine animals off the Swedish coast. In 1969 the Swedish National Poisons and Pesticides Board recommended that aldrin and dieldrin should be banned completely, and that the agricultural use of DDT should be banned for two years (Anonymous, 1969a). Various other countries have banned aldrin and dieldrin. In Britain, the Association of Public Analysts reported that lard contains an undesirably high proportion of pesticide residues, and that residues in apples, brassicas and potatoes exceed the recommended maximum limit of 0.1 p.p.m. (Anonymous, 1969b). The World Health Organisation has also shown concern about pesticide residues in foods (WHO/FAO, 1969).

In view of these harmful effects of chemical insecticides, alternative means for the control of pests are being sought (A.R.C., 1970). These centre on the concept of 'biological control' i.e. using the natural

enemies of the pest to control it (see Norris (1971) and Burges and Hussey (1971)). The present study was undertaken in an attempt to assess whether TIV has any potential value for use as an agent of biological control against leatherjackets.

Several microbial parasites of leatherjackets, other than TIV, are known, viz. a nuclear polyhedrosis virus (Rennie, 1923), a rickettsia (Müller - Kögler, 1958), a fungus (Coulson, 1962), a trypanosome (Vickerman, 1960), a haplosporidian (Huger, 1961) and a gregarine (Steinhaus and March, 1962). In addition, three fungi have been found affecting crane-flies (Koval and Savchenko, 1967). Other documented natural enemies of Tipula spp. are the insect, Siphona (= Bucentes) geniculata (de Geer) (Tachinidae : Diptera) (Rennie, 1912), the nematode, Mermis albicans (Ahmed, 1968) and the following predators: starlings (in discussion after Dunn, 1966), meadow pipits (Coulson, 1962) and spiders (Freeman, 1964). Carabid beetles have been found to feed upon leatherjackets in laboratory tests (Dunn, 1966; Ahmed, 1968) and it is thought that they may be important predators of some leatherjacket species (Freeman, 1967). It may be that any one, or a combination, of the above parasites and predators of leatherjackets might be suitable as agents of biological control.

There are several examples where insect pests have been successfully controlled with viruses. In Canada the European pine sawfly, Neodiprion sertifer (Geoffroy) (Bird, 1953), and the European spruce sawfly, Diprion hercyniae (Hartig) (Bird and Burk, 1961) have been controlled by NPVs; in South Africa a NPV has been used to control the wattle bagworm,

Kotochalia junodi (Heylaerts) (Ossowski, 1957); in the U.S.A. the alfalfa caterpillar, Colias eurytheme (Boisduval) (Thompson and Steinhaus, 1950) and the cabbage looper, Trichoplusia ni (Hübner) (Hall, 1957), have been controlled by NPVs; and in New Zealand the small cabbage white butterfly, Pieris rapae (Linnaeus) has been controlled by a granulosis virus (Kelsey, 1958).

B. SCOPE OF THE WORK

The work described in this thesis was concerned with Tipula iridescent virus (TIV) infections in the insect Tipula oleracea. The experiments were designed to assess whether TIV might have any potential value as a biological control agent. Attempts were made to discover how TIV is transmitted in nature, because a knowledge of the mode of transmission is necessary before the most effective means of applying the virus in the field can be determined. The effects of temperature on the infectivity of the virus and on the rate of development of the disease were studied to determine how the disease develops at different times of the year, thus indicating the most favourable time for application of the virus for effective control.

MATERIALS AND METHODS

Collection of larvae

Most of the original insect material was obtained from the College Estate, Auchincruive, Ayr, Scotland, by watering St. Ives fluid (Appendix 1) onto the soil at a rate of approximately 0.5 l/m^2 (Barnes, 1941). The active ingredient is orthodichlorobenzene, which brings the leatherjackets to the surface. The insects are in no way permanently damaged, and approximately 80% of a leatherjacket population can be obtained by this method. The larvae of the different species are virtually indistinguishable (Lovibond, 1937), but upon rearing the collected insects to adults they were found to be a mixture of T. paludosa and T. oleracea. In subsequent generations there was a higher mortality among the larvae of T. paludosa than those of T. oleracea in the laboratory, so the former were discarded, and the latter were used for the experiments.

Rearing of larvae

The method of rearing larvae was based upon that of Laughlin (1958). The larvae were kept in sand, which had been collected from a river bank, and sterilised by autoclaving at 121° for 30 min. Each progeny group from a pair of adults was kept in a separate plant pot of 178 mm diameter, and the pots stood in trays, containing about 10 mm depth of water. The larvae were reared at room temperature, and fed with dried, powdered grass, which had been sterilised by autoclaving at 121° for 15 min.

The first pupae to be formed were kept at 5° for several days to retard their development, as the males tend to pupate and therefore emerge before the females, and may all die before the females have emerged. The pupae were kept individually in universal bottles, each of which had holes

punched in the lid to allow the passage of air; each bottle contained a moistened filter paper. When the adults emerged, males and females were paired and mated. A male and a female from the same progeny group were never mated together in order to reduce inbreeding.

The technique of Stewart, R.M. (personal communication) was used for mating the adults. This involved confining the pair beneath an inverted Kilner jar, standing on a 90 mm diameter Petri dish, containing 1% 'Ionagar' No. 2 (Oxoid Ltd.) (Fig. 1). After coition the female laid her eggs in the agar (Fig. 2), which served as an ideal medium for keeping the eggs moist. The first eggs to be laid were kept at 5° to slow down their development, until sufficient plates of eggs for the next generation had been obtained. The mean time from laying to hatching of T. oleracea eggs has been found to be 6 days at 21° (Laughlin, 1960), and 6.18 days at 20° (Neats, 1967). A preliminary experiment indicated that they would survive at 5° for at least 3 weeks.

When sufficient plates of eggs had been laid, they were transferred to room temperature to hatch. This process ensured that the ages of all the larvae of one generation were within a few days of each other. Using this technique, the generation time of the insect could be adjusted to almost exactly 10 weeks. When a plate of eggs hatched, the whole mass of agar containing the young larvae was transferred to the surface of a pot of sand.

After 9 generations in the laboratory, the stock was split into 2 lines. The tenth generation was bred from the ninth, as usual, then after a further 5 weeks, the eleventh generation, also bred from the ninth, was hatched. This was achieved by slowing down the development of pupae

Fig. 1. Mating crane-flies

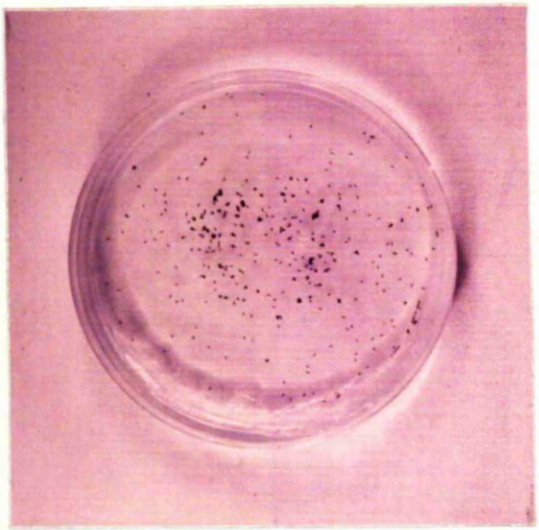


Fig. 2. T. oleracea eggs laid in agar

and eggs at 5°. By this means two staggered lines of insects were obtained, with a generation available for experiments every 5 weeks.

Stressing of larvae

Much work in insect virology has been hindered by the sudden outbreak of a virus infection in an insect population that was supposedly healthy. It is suspected that in many instances the viruses were already present in a latent form, and that some stress factor caused them to become overt (Steinhaus, 1958; Tanada, Tanabe and Reiner, 1964).

In an attempt to detect any latent virus infections in the T. oleracea stock, the first 2 generations bred in the laboratory were subjected to various stress factors. No such latent infection was detected, and experimental work was commenced with the third generation. Some of the stress factors used have stimulated latent infections into activity in other insects. These were:-

1. High temperature (Hukuhara and Aruga, 1959). The larvae were exposed to a temperature of 37° for 48 h.
2. Low temperature (Aruga, 1957) followed by high temperature. The larvae were exposed to a temperature of 0° for 48 h, and then 30° for 48 h.
3. Starvation (Steinhaus and Dineen, 1960). The larvae were given no food for 7 days.
4. Feeding protein (Tanada, 1954) and amino acids. The larvae were fed B-lactoglobulin, ovalbumen, DL-alanine, L-glutamic acid or L-tyrosine, mixed 10% w/w with dried grass.
5. Feeding toxic chemicals (Aruga and Hukuhara, 1960). The larvae were fed mercuric chloride or sodium fluoride mixed 10% w/w with dried

grass, or 40% formaldehyde, 10-volume hydrogen peroxide, or thioglycollic acid mixed 10% v/w with dried grass.

6. Irradiation with ultra-violet (UV) light (Steinhaus and Dineen, 1960). The larvae were irradiated by means of a UV lamp, at a distance of about 30 cm, for approximately 1 h daily, over 7 days.

Subjection of groups of second-generation larvae to these stress factors resulted in the mortalities indicated in Table 1.

None of the larvae which died developed iridescence, and no polyhedra of the NPV were detected in Giemsa-stained smears of their haemolymph, so it was concluded that the field-collected larvae were probably free of latent virus infections. All the insects used in the experiments were descended from the survivors of these stressed larvae. The stress factors possibly assisted in the production of a more vigorous insect stock by eliminating the weaker individuals.

Experimental larvae

Unless otherwise stated, all experimental larvae were kept in individual petri dishes at 20°. The individual petri dishes were necessary because leatherjackets use their mandibles as offensive weapons, and launch fatal attacks upon each other (Freeman, 1966). Sterile petri dishes were used, and a sterilised filter paper was placed in each dish. The larvae were fed with sterilised, dried, powdered grass, and it and the filter paper were kept moist with the salts and vitamins solution of David and Gardiner (1965a) (Appendix 2).

The sterile materials were used in an attempt to reduce mould growth, which proved to be a problem from time to time. Some moulds can produce

Table 1. Effects of stress factors upon leatherjackets.

Stress Factor	No. dead/ No. treated	Percentage Mortality
high temperature (37°)	153/153	100
feeding L-tyrosine	10/10	100
feeding mercuric chloride	10/10	100
feeding sodium fluoride	10/10	100
feeding formaldehyde	10/10	100
feeding thioglycollic acid	10/10	100
feeding B-lactoglobulin	9/10	90
UV irradiation	46/58	79
feeding L-glutamic acid	7/10	70
feeding hydrogen peroxide	7/10	70
starvation	32/47	68
feeding DL-alanine	6/10	60
low (0°)/high (30°) temperature	66/114	58
feeding ovalbumen	4/10	40
controls	5/22	23

toxins which are harmful to insects (Beard and Walton, 1969). A brief experiment was performed to determine whether cycloheximide would be of any value in inhibiting mould growth, but it was found that the concentrations of cycloheximide which were required to inhibit mould growth also caused the deaths of the leatherjackets. The insecticidal properties of cycloheximide have also been observed by Fytizas (1968). Because of the growth of moulds in the dishes, and the accumulation of excreta, the insects were placed in clean dishes at intervals of not more than 4 weeks.

Injection of larvae

When larvae were divided into groups, e.g. to receive different doses of TIV, the members of the progeny groups were randomly distributed among them, in order to minimise the effects of any genetic differences between the progeny groups.

Larvae to be injected were anaesthetised with ether (Bergold, 1951) by placing them in the apparatus shown in Fig. 3 for approximately 10 min. They were then rinsed in water, 2.5% 'Dettol', and twice again in water to clean their skin surfaces.

The injections were performed with a micrometer syringe fitted with a 0.25 mm (outside diameter) needle. The insect was held between the fingers of the left hand, and the right hand was used to manipulate the micrometer; the needle was inserted dorsally into the haemocoel, about one third of the length of the larva from its anterior end. Each injected insect received 0.005 ml material, unless otherwise stated. Before withdrawing the needle, it was held inside the haemocoel for a few seconds to allow

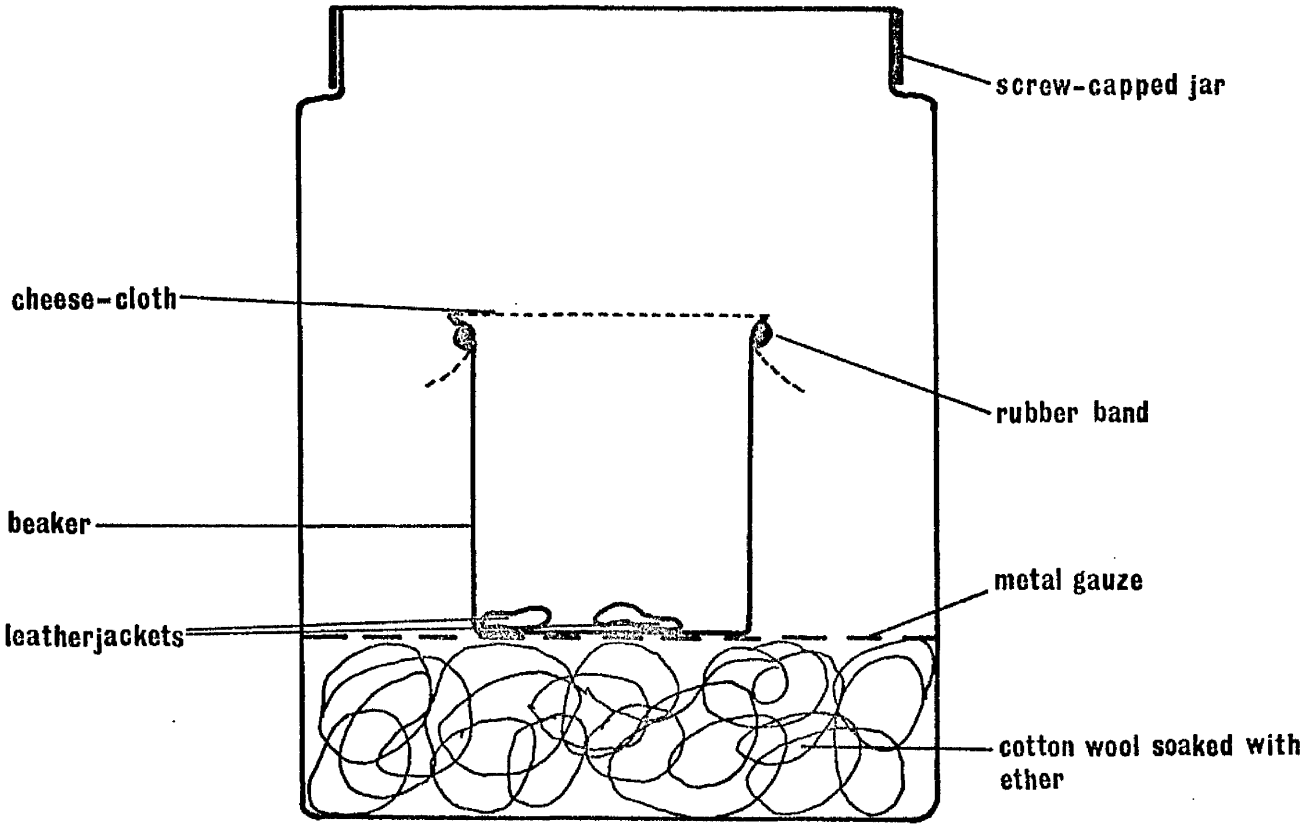


Fig.3. Apparatus for anaesthetising leatherjackets.

the injected material to be dispersed throughout the insect because when the needle was withdrawn bleeding occurred. It was shown (Experiment 3) that this bleeding did not result in any significant loss of virus from the insect.

TIV for injections was suspended in insect saline with antibiotics (ISA). The insect saline was that of Clarke (Appendix 3) (Hale, 1965). The antibiotics were incorporated in an attempt to reduce bacterial infections being set up at the time of injection (Bird, 1961; Smith et al, 1961). All injected larvae were in the fourth instar, unless otherwise stated.

Purification of TIV

TIV was purified according to the method of Bird (1961) and Thomas (1961) who subjected the virus to alternate cycles of low- and high-speed centrifugation. Infected larvae were ground in distilled water or 0.85% NaCl (saline) using a pestle and mortar, and large debris was removed by centrifuging at 600 g for 5 min. Impure TIV was removed from the supernatant by centrifuging at 7,600 g for 20 min. The resulting iridescent pellet was covered with a layer of impurities, which was removed by washing gently, using a pasteur pipette. The pellet was resuspended in saline, and subjected to 4 more cycles of low-/high-speed centrifugation. The resulting TIV preparation was reasonably free from impurities.

Further purification, if desired, was carried out by centrifuging in a sucrose gradient (Smith et al, 1961). A sucrose gradient of 5 - 35% w/w in phosphate-buffered saline (PBS) was employed, and was centrifuged at 6,150 g for 30 min. The band of TIV was removed by pipetting, and the

virus was sedimented by centrifuging at 14,900 g for 40 min. It was finally washed in distilled water.

Preparation of antiserum

Antiserum against TIV was prepared by the method of Cunningham and Tinsley (1968). A preparation of TIV was purified in a sucrose gradient, and suspended in distilled water. The concentration of virus was found by weighing, then it was suspended in saline at a concentration of 1 mg/ml. This was incorporated in an equal quantity of Freund's complete adjuvant.

Two rabbits each received 3 injections of TIV at weekly intervals. The first 2 injections consisted of 1.5 ml virus-adjuvant mixture injected subcutaneously, and the third of 1.0 ml TIV suspension in saline injected intravenously. A blood sample had been collected from each rabbit before the injections were performed. Blood samples were taken 2 weeks after the final injection, and then at weekly intervals over a period of 4 weeks. The sera were stored at -16°.

Neutralisation test

The presence of antibodies against TIV in the sera was demonstrated by means of the neutralisation test. Decimal dilutions of TIV in PBS were prepared to 10^{-7}. The second and final serum from each rabbit was tested by mixing 0.2 ml serum with 0.2 ml of each dilution of TIV. There were two controls; in one the antiserum was replaced by normal rabbit serum, and in the other by PBS. All mixtures were incubated at 37° for 2 h, and then tested for infective virus by injection into leatherjackets. Three leatherjackets were used to test each mixture. The results indicated that each antiserum neutralised TIV infectivity by a factor of approximately 10^{5}.

Tube precipitation test

The method was based upon that of Cunningham and Tinsley (1968). Doubling dilutions of serum were prepared with saline. Each dilution was transferred to a Dreyer tube, and an equal volume of TIV in saline (0.025 mg/ml, measured by UV spectrophotometry) was added. The tubes were incubated in a water-bath at 50° and read after 5 h; they were then removed from the water-bath and read again the next morning. The serum tested had a titre of 1/256.

A serum dilution of 1/64 was then used to find the optimum dilution of antigen. Doubling dilutions of TIV in saline (2.5 mg/ml) were prepared, and the end-point was found to be 1/256. A 1/64 dilution (0.039 mg/ml) was therefore used to titrate all the sera; the titres of most were found to be 1/512, and of some, 1/256.

Latex test

The methods used were those of Abu Salih, Murant and Daft (1968).

a) Sensitisation of latex particles

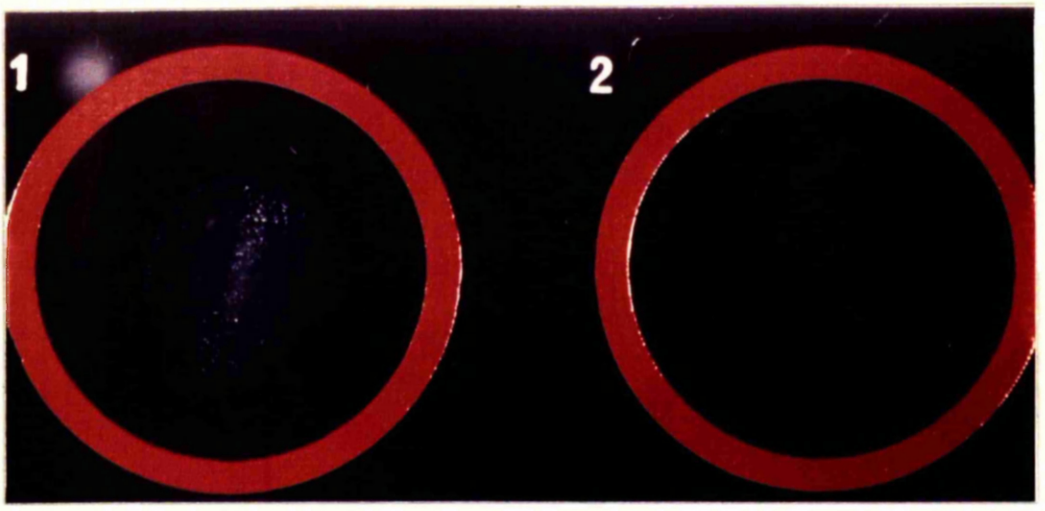
One volume of antiserum against TIV was diluted with 9 volumes distilled water, and 10 volumes of saturated ammonium sulphate solution were added dropwise. The mixture was allowed to stand for 10 min, and was then centrifuged at 5,000 *g* for 30 min. The pellet was suspended in the same volume of water as before, and precipitated again with ammonium sulphate. The precipitate was resuspended in 3 - 4 volumes saline, and dialysed against saline for 2 h at room temperature. It was dialysed for a further 2 h against fresh saline, and then made up to 10 volumes with saline, giving a concentration of 1/10 for the original globulins. From this, further dilutions of the globulins were prepared

in tris (tris (hydroxymethyl) methylamine) HCl buffer (pH 7.2).

A suspension of polystyrene latex particles (diameter 810 nm; approx. 1.5×10^{10} particles/ml) was diluted 1/15 with 0.9% w/v NaCl. One volume of the diluted globulins and one volume of the diluted latex suspension were incubated together for 30 min at room temperature, and then centrifuged at 5,000 g for 30 min. The pellet was resuspended in half a volume of tris HCl containing 0.02% polyvinyl pyrrolidone (PVP), and then centrifuged at 4,000 g for 30 min. The final pellet was suspended in half a volume of tris HCl + PVP + 0.2% sodium azide, and stored at 5°.

b) Tests.

The tests were carried out on glass plates, painted black on the reverse side. One drop of antigen, suspended in tris HCl + PVP was mixed with 2 drops of the suspension of sensitised latex particles. Controls consisted of 1) tris HCl + PVP plus sensitised latex particles, 2) TIV suspension plus tris HCl + PVP and 3) TIV suspension plus unsensitised latex particles. The plates were gently rocked, and inspected under a 60 W light bulb for clumping of the latex particles, which indicates a positive result (Fig. 4). Abu Salih et al (1968) took their final readings after 10 min, but it was found that in some tests positive results did not appear until after this time, so the final readings were made after 40 min. Tests carried out using suspensions of latex particles sensitised with various dilutions of globulins and suspensions of various dilutions of TIV indicated that the optimum dilution of globulins for sensitising the latex was about 1/600.



positive negative

Fig. 4. Latex test results

Ultraviolet spectrophotometry

UV spectrophotometry was used to measure concentrations of TIV suspensions, and, in Experiment 24, UV spectra of TIV at different pHs were determined. The measurements were made with a Unicam SP500 spectrophotometer fitted with a hydrogen lamp, and were corrected for light scattering by measuring the optical densities at several wavelengths greater than 320 nm, where the total optical density is due to scattered light (Kleczkowski, 1968).

For large virus particles, $\tau = \frac{C}{\lambda^n}$ where τ = optical density

C = "scattering constant"

λ = wavelength.

$$\log \tau = \log C - n \log \lambda$$

A plot of $\log \tau$ v $\log \lambda$ gives a straight line, with slope = $-n$.

Substitute n in $D = R - S \left(\frac{320}{\lambda}\right)^n$

where D = optical density due to absorption

R = spectrophotometer reading

S = optical density at 320 nm.

Concentrations of TIV suspensions in distilled water were determined by evaporating to dryness over calcium chloride in a vacuum desiccator. The concentrations of unknown suspensions could then be determined from the formula:

$$\text{Concentration of TIV} = \frac{\text{corrected optical density at 260 nm x dilution factc}}{E \begin{smallmatrix} 0.1\% \\ 1\ cm \end{smallmatrix}}$$

where E = extinction coefficient at 260 nm (see Fig. 28).

Day and Dudzinski (1966) used UV spectrophotometry to measure concentrations of SIV.

Detection of TIV

TIV was generally detected by injection into leatherjackets, most of which developed iridescence if they received an infective dose. Other possibilities were 1) the larva died without iridescence developing, 2) the larva pupated, and the pupa became iridescent, 3) the larva pupated and died, and 4) the larva pupated, and gave rise to an adult. The presence of TIV infection in dead larvae, pupae and adults could be detected by one of three methods:

a) Dissection of the insect, when iridescence of the fat body might be observed.

b) Grinding the insect in distilled water, followed by centrifugation at 600 g for 5 min, then centrifugation of the supernatant at 7,600 g for 20 min, and inspecting the resultant pellet for iridescence. This operation was performed in polystyrene tubes which are clear, enabling a small iridescent pellet to be easily seen. Day and Mercer (1964) used a similar technique to detect SIV in G. mellonella larvae which had been inoculated with the virus, but had not developed iridescence.

c) Grinding the insect in 1 ml tris HCl + PVP, followed by centrifugation at 600 g for 5 min in a polystyrene tube, then performing a latex test on the supernatant. The latex test was found to be slightly more sensitive than the centrifugation technique for detecting TIV (Experiment 4).

Membrane filtration of TIV

Filtration through cellulose nitrate membranes was carried out to free preparations of TIV from bacterial, fungal and other contaminants.

The virus was suspended in deionised distilled water, and filtered through membranes with progressively smaller pore sizes, finishing with a 0.2 µm membrane. The membrane was held in a Sartorius filter adapter V25, which was sterilised by autoclaving at 121° for 15 min, with the membrane in place. Before use, each membrane was coated with serum to prevent adsorption of the virus (Ver, Melnick and Wallis, 1968). The serum ('Rabbit serum no. 1', Burroughs Wellcome & Co.) was filtered successively through sterile 2.0 µm, 0.8 µm and 0.2 µm membranes before use. Membrane filtration was used by Tanada and Tanabe (1965) in the final stage of purification of TIV. The efficiency of this technique was assessed in Experiment 6.

EXPERIMENTS

A. PRELIMINARY EXPERIMENTS

Experiment 1. Characterisation of the virus.

a) Electron microscopy

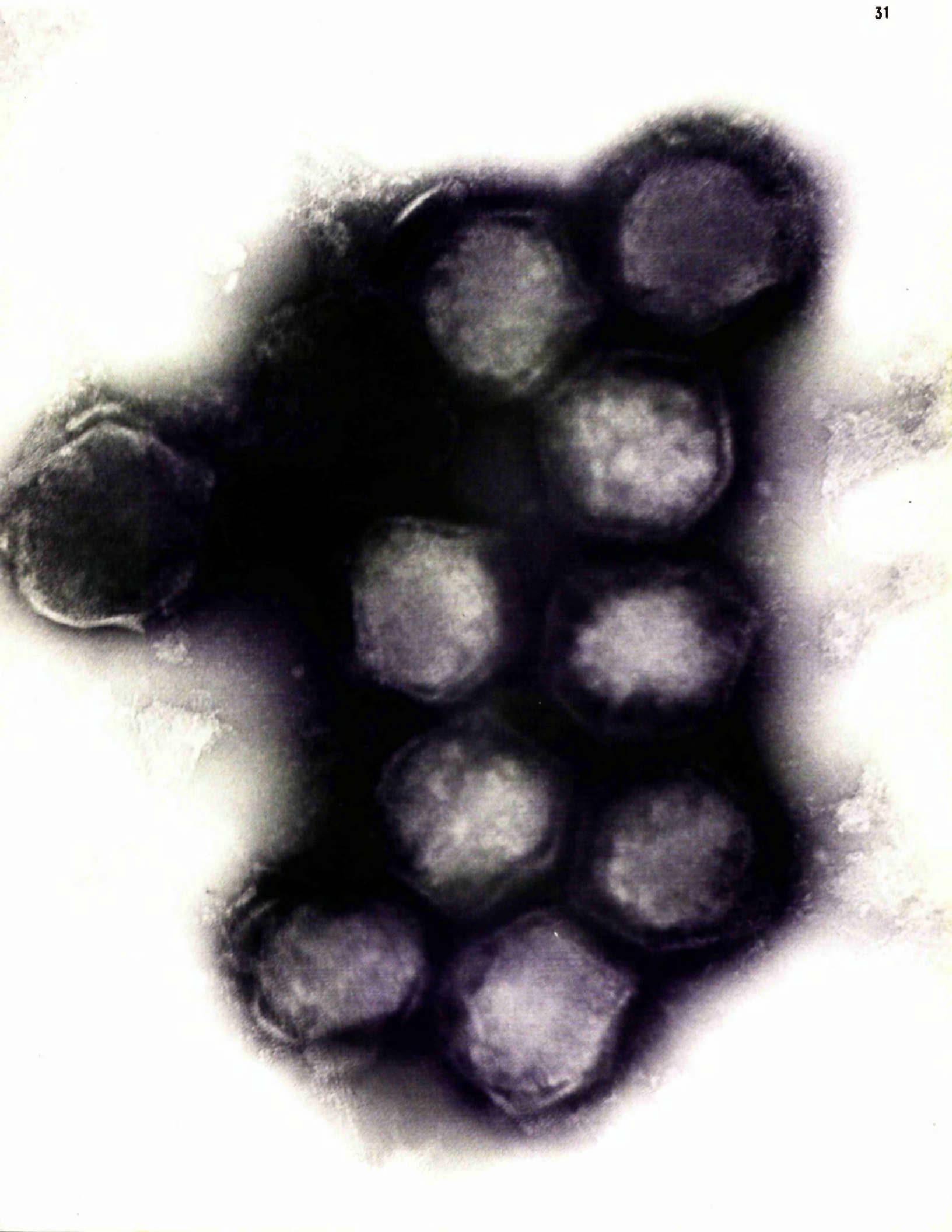
Many workers have examined TIV in the electron microscope, and a number of electron micrographs have been published, e.g. by Williams and Smith (1958), Glitz et al (1968) and Wrigley (1970). It is agreed that the TIV particle is an icosahedron bounded by 2 membranes, but there is disagreement over the number of protein subunits of which these membranes are composed and over the diameter of the virion (see General Introduction).

A sample of the virus used in the present investigations was suspended in distilled water and mixed with 2% ammonium molybdate. A drop was applied to a formvar-coated specimen grid stabilised with a thin layer of carbon. The droplet was removed with a filter paper point, leaving a thin film of specimen, which was examined in a Philips EM300 electron microscope at operating voltage 60 Kv. The appearance of the virus (Fig. 5) is similar to that of TIV in published electron micrographs, the double membrane at the periphery of the virion being clearly visible. The average diameter of the virions is 130 nm, which is in agreement with the measurements of Williams and Smith (1957) and Thomas (1961) for TIV.

b) Production of iridescence

Centrifugation of the virus, e.g. at 7,600 g for 20 min, resulted in the formation of an iridescent pellet, as described by Williams and Smith (1957); in reflected light it was generally blue in colour (Fig. 6), but sometimes appeared purple or green, and in transmitted light it

Fig. 5. Electron micrograph of the virus used for the present investigations. x 336,000.



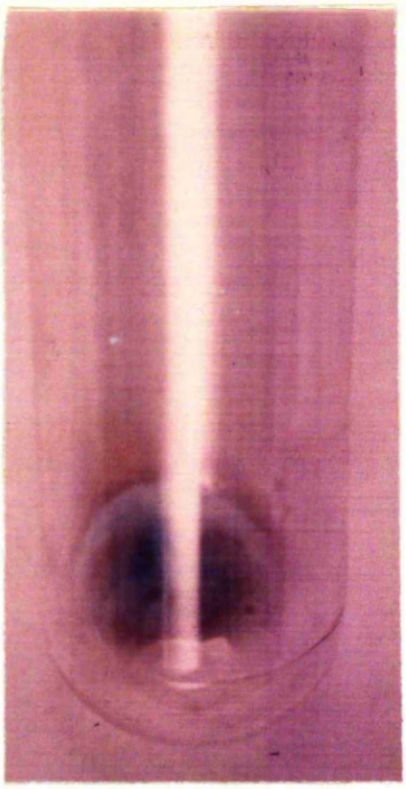
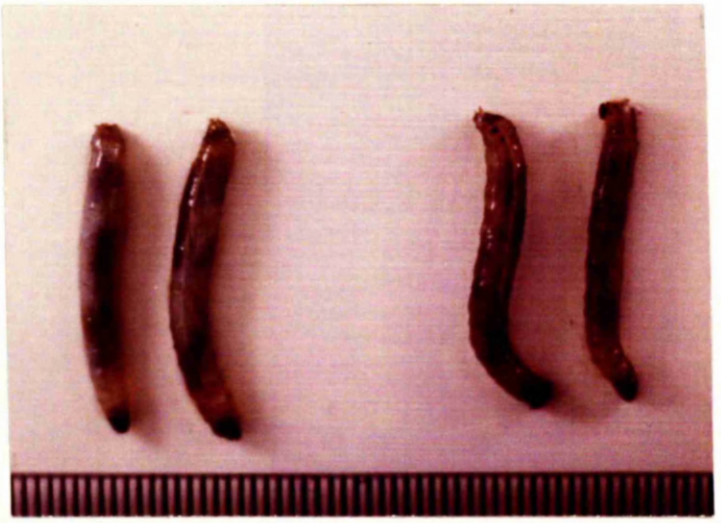


Fig. 6. Iridescent pellet of virus



virus-infected not infected

Fig. 7. Fourth-instar T. oleracea larvae

Scale : mm.

appeared orange. Injection of the virus into leatherjackets resulted in a characteristic TIV infection, as described by Xeros (1954). Iridescence developed in the fat body, and this could be observed through the skin after several days (Fig. 7).

Discussion

The virus used in the present investigations was isolated from a leatherjacket displaying iridescence. The morphology and diameter of the virion, and the iridescence displayed by virus pellets and the infected insects indicate fairly conclusively that this virus is Tipula iridescent virus.

Experiment 2. Assay of TIV.

Introduction

A common method of measuring the concentration of a pathogen is to determine the medial lethal dose (LD50), the results being analysed by one of several possible methods, e.g. by the method of Reed and Muench (1938) or by probit analysis (Finney, 1952). An alternative method is to measure the time for a response to be elicited in the host after inoculation with an unknown dose of the pathogen, and to estimate the dose from a standard curve prepared by measuring the response times for known doses.

Meynell and Meynell (1958) carried out a survey of the literature, and observed that for a number of host/parasite systems the mean response time is linearly related to log dose. They suggested that the organisms increase exponentially at the same rate at all doses, and that the response occurs when the total number of organisms reaches a critical figure.

This model fits the results of Aizawa (1959), who showed that both a 10^{-5} dilution of silkworm NPV and the undiluted preparation resulted in an identical rate of increase in the inoculated insect.

Gard (1940) measured the survival time of mice inoculated with encephalomyelitis virus, and prepared a standard curve using the reciprocal of the harmonic mean survival time:

$$\frac{1}{T} = \left(\frac{1}{t_1} + \frac{1}{t_2} + \frac{1}{t_3} \ldots\ldots \frac{1}{t_N}\right) \times \frac{1}{N}$$

where t_1, t_2 etc. are the individual survival times for N hosts in the dose-group. The reciprocal transformation of the data tends to equalise the variance of the responses over the whole range of doses. Smith and Westgarth (1957) and Bauer (1960) also assayed viruses in mice by measuring survival time, and Dougherty, McCloskey and Stewart (1960) assayed the psittacosis agent by measuring the survival time of embryonated eggs inoculated with the agent. Survival time of the host can also be used to compare the virulence of different virus strains (Reeve, 1969).

Among the insects there are several reports of decreasing response times with increasing inocula of infective agent. Increasing dosages of NPV result in shorter survival times in the sawflies, Neodiprion sertifer (Krieg, 1955) and Neodiprion swainei (Middleton) (Smirnoff, 1961), the western oak looper, Lambdina fiscellaria somniaria (Hulst) (Morris, 1962), the bollworm, Heliothis zea (Boddie), and the tobacco budworm, Heliothis virescens (Fabricius) (Ignoffo, 1965), and Trichoplusia ni (Canerday and Arant, 1968). Aizawa (1953) observed that lower concentrations of NPV inoculated into silkworm pupae resulted in longer latent periods, and Henry and Jutila (1966) observed that the time for the appearance of polyhedra in the grasshopper, Melanoplus sanguinipes (Fabricius), was influenced by the size of the virus inoculum. In aphids, the larger the amount of virus in the infection feed, the more rapidly the insects become inoculative, e.g. strawberry virus 3 in Capitophorus fragariae (Theobald) (Prentice and Woollcombe, 1951) and sowthistle yellow vein virus in Hyperomyzus lactucae (Linnaeus) (Sylvester, Richardson and Behncken, 1970).

Several workers have assayed infective material in insects by measuring a response time. Maramorosch (1955a) assayed aster yellows virus by measuring the time for its vector, Macrosteles fascifrons (Stål) to become inoculative. The time for Drosophila to develop CO_2-sensitivity has been used to assay the sigma virus (L'Héritier, 1958). Ignoffo (1964) suggested that measurement of the median lethal time could be used to estimate the NPV of T. ni. Whitcomb, Jensen and Richardson (1966 assayed the agent of Western X-disease by measuring the time for the leafhopper, Colladonus montanus (Van Duzee), to become inoculative.

The response time method was investigated as a possible technique for assaying TIV, the incubation period (time for the insect to develop iridescence) and the survival time being considered. The response time method was compared with the usual method of LD50 determination and with serological methods of assay.

Methods

A preliminary assay was carried out on a suspension of TIV in ISA by injecting larvae with decimal dilutions, each dilution being injected into 3 larvae; the results indicated that the LD50 was approximately 10^{-5}. The following dilution series was then prepared; $10^{-0.301}$, 10^{-1}, $10^{-1.301}$, 10^{-2} ..... to 10^{-6} and each dilution was injected into 30 larvae. The results of this assay indicated that the dilution series did not extend to the LD50, so a further set of dilutions was prepared from 10^{-5} to 10^{-8}, and each of these was injected into 30 larvae. The TIV suspension was stored at 5° between the two sets of inoculations, which were 16 weeks apart; it was assumed that there was little alteration in infectivity

of the virus during this time (see Experiment 23). Latex tests and tube precipitation tests were carried out on decimal dilutions of the TIV suspension.

Results and Discussion

a) Median lethal dose (LD50)

The numbers of insects becoming infected with TIV are given in Table 2. Some did not develop iridescence until after they had pupated, and some died before iridescence developed, the infection being detected by the latex test. The dosage-mortality relationship is indicated in Fig. 8; the LD50 was computed from this by probit analysis and found to be $10^{-5.681}$, with 95% confidence limits of $10^{-5.498}$ and $10^{-5.874}$. Bucher (1956) pointed out that the slope of the dosage-mortality curve is a measure of the variation in resistance to treatment in the population. In the present experiment the slope was 0.86 and there appeared to be wide variation in larval resistance to TIV, some insects becoming infected by the 10^{-7} dilution of TIV, while one did not become infected by the 10^{-3} dilution.

b) Incubation period

The relationship between mean incubation period and log. dose (Fig. 9) appears to be linear at the higher dilutions, but not so at the lower dilutions. This may be due to the saturation of receptor sites for virus on the host cells at the lower dilutions (Heynell and Heynell, 1958). The cells at the surface of the fat body become infected before those in the interior (Kanyuka and Pronina, 1970); when all the receptor sites on the susceptible cells have taken up virus, increasing the dosage will not result in shorter incubation periods.

Table 2. Numbers of insects becoming infected after injection with dilutions of TIV.

Dilution of TIV	First assay			Second assay			Percentage becoming infected
	Early deaths*	Infected	Not infected	Early deaths*	Infected	Not infected	
10^{0}	2	28	0	-	-	-	100.0
$10^{-0.301}$	3	27	0	-	-	-	100.0
10^{-1}	0	30	0	-	-	-	100.0
$10^{-1.301}$	1	29	0	-	-	-	100.0
10^{-2}	1	29	0	-	-	-	100.0
$10^{-2.301}$	1	29	0	-	-	-	100.0
10^{-3}	0	29	1	-	-	-	96.7
$10^{-3.301}$	1	26	3	-	-	-	89.7
10^{-4}	3	26	1	-	-	-	96.3
$10^{-4.301}$	2	25	3	-	-	-	89.3
10^{-5}	0	19	11	3	24	3	75.4
$10^{-5.301}$	0	21	9	4	21	5	75.0
10^{-6}	4	17	9	1	7	22	43.6
$10^{-6.301}$	-	-	-	4	9	17	34.6
10^{-7}	-	-	-	5	3	22	12.0
$10^{-7.301}$	-	-	-	2	0	28	0.0
10^{-8}	-	-	-	5	0	25	0.0

* The 'early deaths' comprise those insects which died and were latex-negative up to two days before iridescence began to appear in that dose-group. These were discounted when calculating the percentage of insects becoming infected.

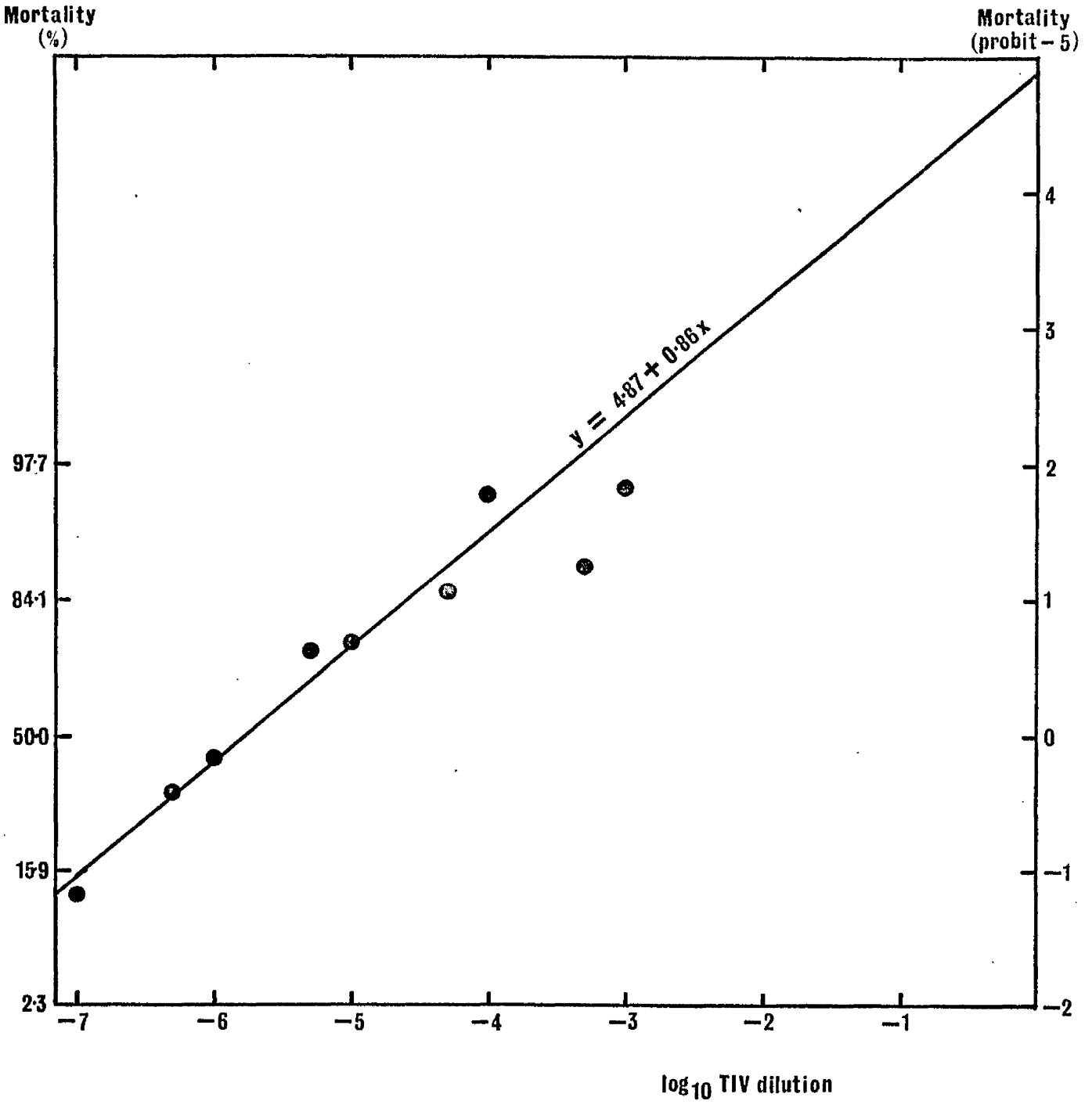


Fig.8. Log dose – mortality relationship.

A straight line was fitted between the 10^{-4} and the 10^{-7} dilutions (Fig. 9) and, assuming that this represents an exponential increase, an estimate of the doubling time of TIV was made as follows:

Let the estimated line be

$$y = a + bx$$

where x = log dose

y = mean incubation period

$$y = 1.6633 + 1.5396x$$

$$y_1 = a + b \log \text{dose}$$

$$y_2 = a + b \log 2 \text{ dose}$$

$$y_2 - y_1 = b \log 2 \quad \text{where } y_2 - y_1 = \text{doubling time}$$

$$= 1.5396 \times 0.3010 \times 24 \text{ h}$$

$$= 11.122 \text{ h}$$

95% confidence limits are given by

$$(y_2 - y_1) \pm t_{(n-2),\, 0.05} \sqrt{V(b) \times (\log 2)^2}$$

Doubling time = 11.1 h ± 2.1 h

The mean incubation period at the LD50 = 10.4 days (Fig. 9), which is equivalent to 22.4 doubling times for TIV.

There was a high degree of variability in the results for incubation period, which can be seen in Fig. 10. 93.6% of the variation in incubation period can be accounted for by variations in dose. These curves show the details of which Fig. 9 is a summary.

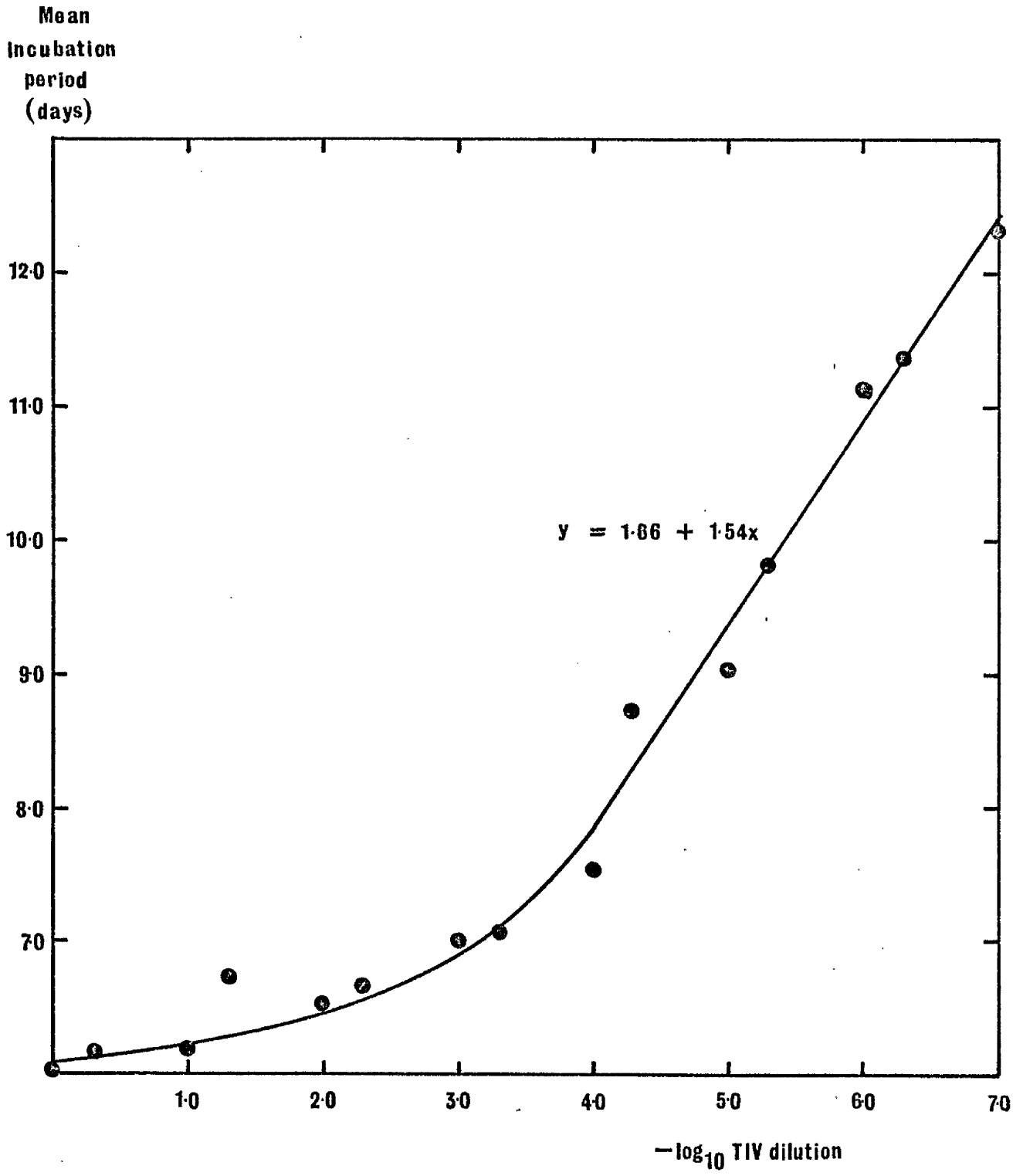


Fig.9. Relationship between log dose and mean incubation period.

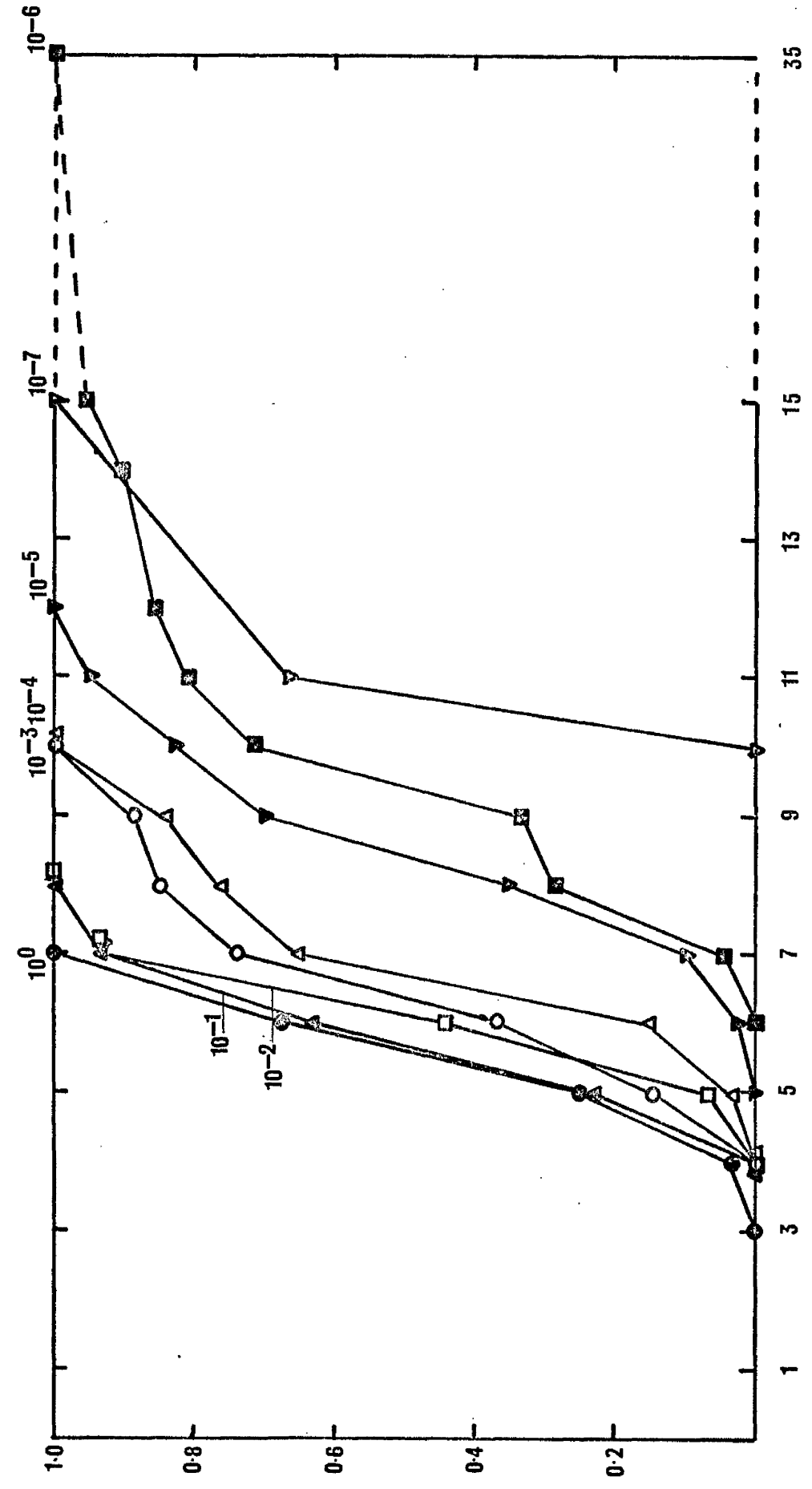


Fig.10. Rates of appearance of iridescence in larvae injected with dilutions of TIV.

Data for decimal dilutions only are shown

c) Survival time

The survival times showed greater variability than the incubation periods, although there was still a tendency for longer survival times with higher dilutions of TIV (Fig. 11). 61.5% of the variation in survival time can be explained by variations in dose. The mean survival time varied from 29.5 days for the undiluted TIV preparation to 61.0 days for the $10^{-6.301}$ dilution. There was virtually no correlation between incubation period and survival time ($r = 0.126$).

d) Preparation of standard curve

It was decided to use incubation period as a response time for assay purposes in preference to survival time because of the greater variability of the latter, and the longer time to obtain results. The reciprocal of the harmonic mean incubation period was plotted against log dose, and a straight line was fitted (Fig. 12a). Using this line, 93.6% of the variation in incubation period can be explained by variations in dose. As mentioned above, the relationship appears to be more nearly linear at higher dilutions of virus, so a second straight line (Fig. 12b) was fitted, between 10^{-3} and 10^{-7}; this line explains 97.6% of the variability. The best line using the data from all the dose-groups was found to be a parabola (Fig. 12c), which explains 97.4% of the variability. It was therefore decided to use the parabola as the standard curve for assay purposes, TIV titres being measured in LD50 units.

e) Comparison of assay techniques

The response time method has several advantages over the usual LD50 determination: no preliminary assay is required to determine a suitable range of dilutions; results are generally obtained more rapidly

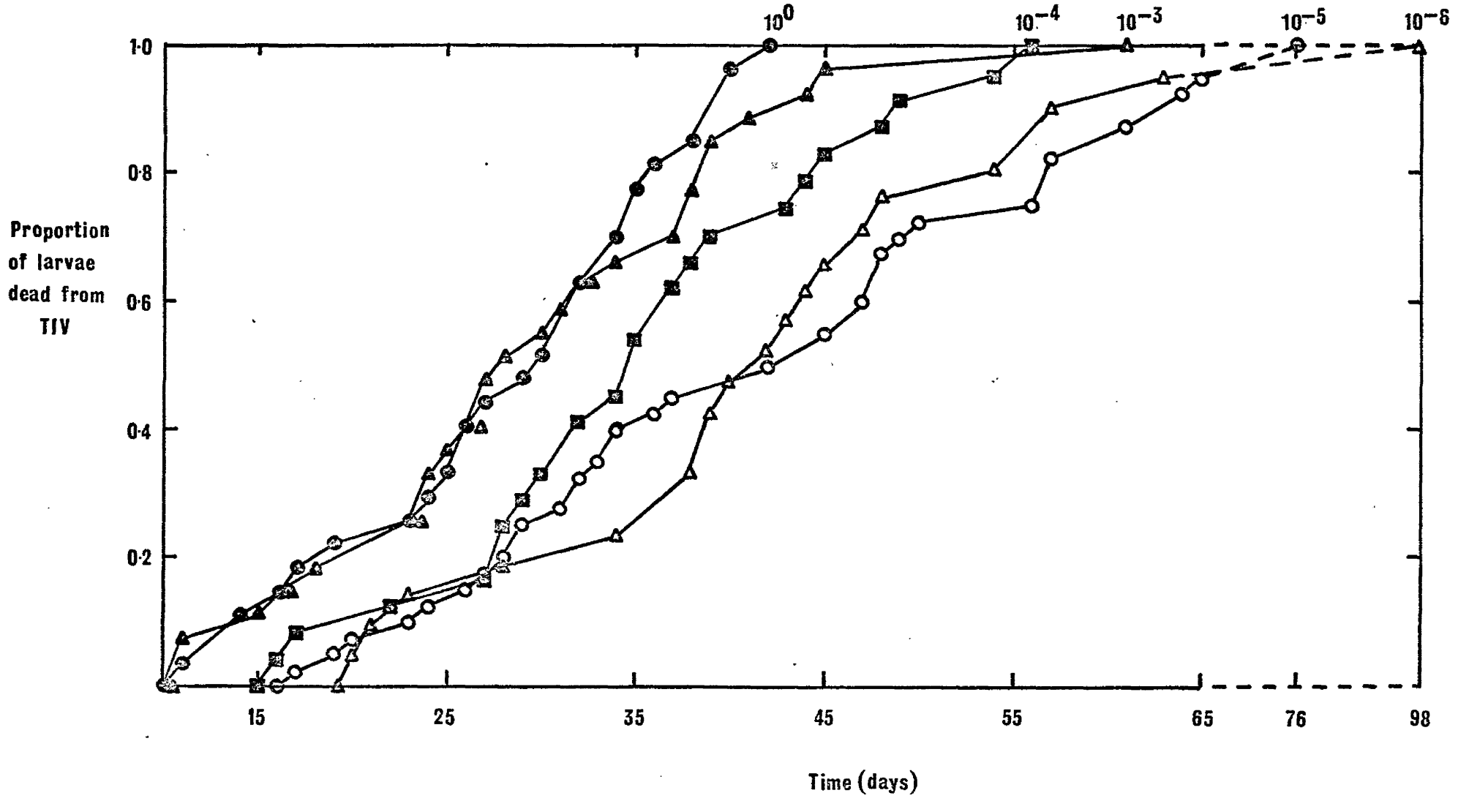


Fig.11. Death rates of larvae injected with dilutions of TIV.

Data for only five dilutions are shown

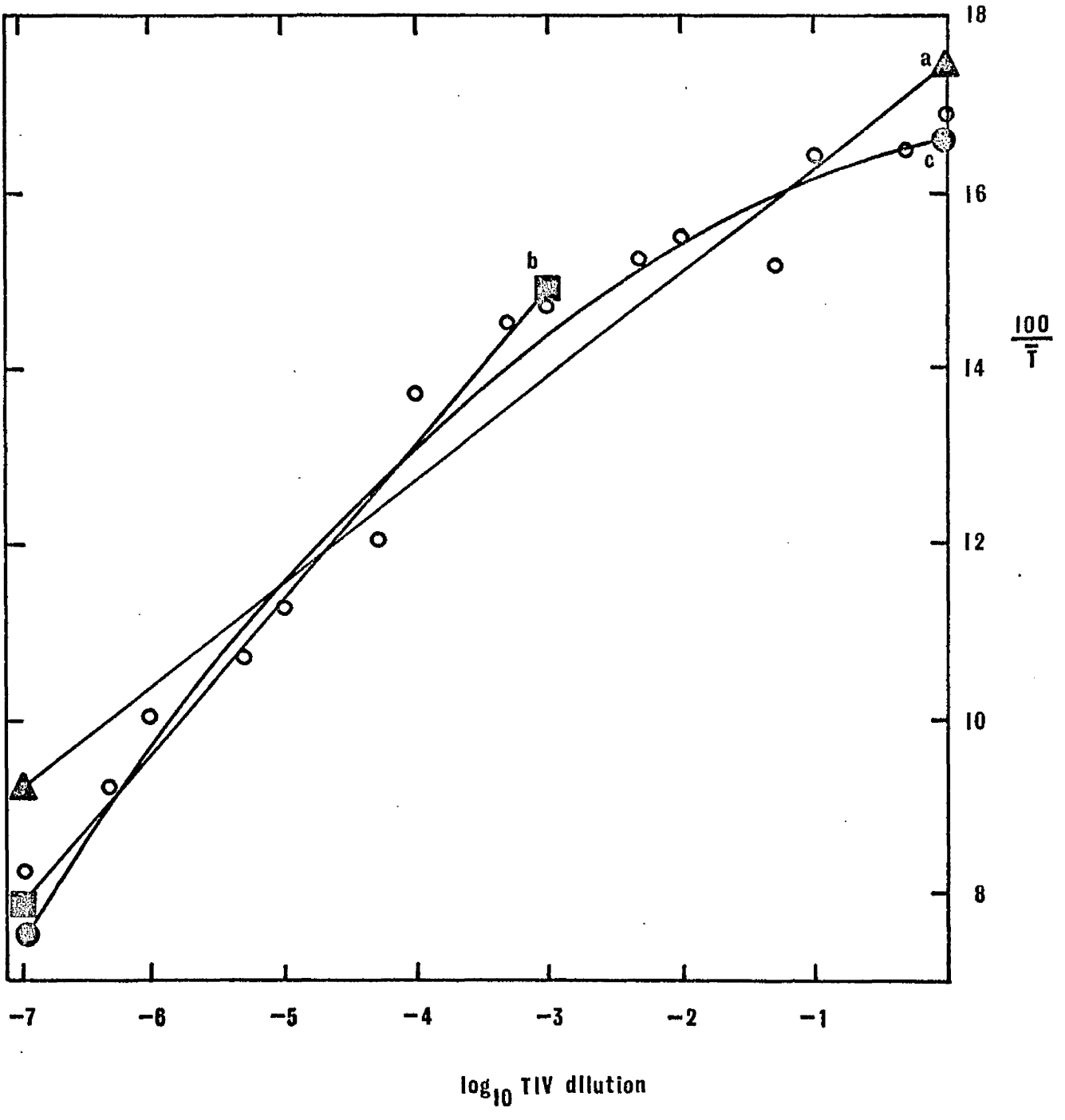


Fig.12. $\frac{100}{\bar{T}}$ v log dose, where $\bar{T}$ is the harmonic mean incubation period in days.

	o o	observed values
a	△—△	$y = 17{\cdot}472 + 1{\cdot}179x$
b	▣—▣	$y = 20{\cdot}177 + 1{\cdot}746x$
c	◉—◉	$y = 16{\cdot}705 + 0{\cdot}326x - 0{\cdot}140x^2$

as incubation periods are shorter with low dilutions; fewer dilutions generally need to be prepared, with fewer errors as a consequence. Glitz et al (1968) observed that TIV is adsorbed onto glass surfaces, which can result in errors during the preparation of dilutions.

TIV was assayed by the response time method on a number of occasions during the present investigations. Two dilutions of the virus were generally assayed, and the mean of the 2 estimates was taken. In some cases the 2 estimates were in almost complete agreement, while in others there were large discrepancies between them. Possible reasons for these discrepancies are loss of virus from the larva when it bleeds after the injection, and variation between larvae in susceptibility to infection, which the slope of the dosage-mortality curve (Fig. 8) suggests is high. An attempt was made to diminish bleeding after injections, but this did not result in shorter or less variable incubation periods (Experiment 3).

It was shown in Experiment 7 that different progeny groups vary in their rate of development, and in Experiment 22 that growth rate is affected by temperature. All larvae for the present experiments were bred at room temperature, which was not constant, thus larvae of the same age in different generations were not always at the same stage of development. In Experiment 23 the TIV titres in the third assay were higher than the second in all 3 cases, which suggests differences between the larvae used for the 2 sets of assays. It would be preferable to breed all insects under conditions of constant temperature.

Probit analysis gave a fairly precise estimate of the LD50 of the TIV suspension; this was based on the data from 270 larvae inoculated

with dilutions widely spaced around the LD50 (10^{-3} to 10^{-7}). If an approximation of the LD50 were known from a preliminary assay, then the same precision could be achieved with fewer larvae inoculated with dilutions more closely spaced around the LD50. The possible sources of error mentioned above for the response time method, i.e. adsorption of TIV onto glass, loss of TIV from the insect during bleeding, and variations in susceptibility to infection between larvae, also apply to the usual method of LD50 determination.

Latex and tube precipitation tests carried out on the TIV suspension gave end-points of 1/2,000 and 1/200, respectively, thus these techniques could only be used for assaying concentrated suspensions of TIV. Serological tests such as these do not necessarily measure the infectivity of a virus suspension, as virions might possibly lose their infectivity without losing their serological specificity. The main advantage of these tests is their rapidity and they could be useful for carrying out a preliminary assay on an unknown TIV suspension if the LD50 were to be determined by the usual method.

Experiment 3. Attempts to diminish bleeding in larvae after injection.

Introduction

One of the reasons postulated for the high degree of variability in incubation period in leatherjackets injected with TIV (Experiment 2) was a loss of virus when the insect bleeds after the syringe needle has been withdrawn. Wittig (1963) suggested sealing wounds in insects caused by injection with a sealing material such as collodion.

Methods

The following materials were used to try to diminish bleeding in leatherjackets: vaseline, lanoline, soft yellow paraffin, collodion and 'New-Skin' (Harwoods Laboratories, St. Helens, Lancs.). Each of these was tested on 50 larvae, which were each injected with identical doses of TIV; 50 control larvae were each injected with the same dose. The vaseline, lanoline and paraffin were smeared over the injection site, and the injection was performed through the smear. The collodion and 'New-Skin' were applied to the wound immediately after the needle had been withdrawn. It was considered that if any of these treatments resulted in significantly shorter incubation periods compared to those in the control larvae, then this would indicate that less virus had been lost during the post-injection bleeding.

Results and Discussion

None of the treatments resulted in incubation periods significantly shorter than the controls (Tables 3 and 4), but the lanoline treatment resulted in significantly longer incubation periods, a result which indicates reduction in the effective dose, possibly by prevention of

Table 3. Results of attempts to diminish bleeding in leatherjackets after injection with TIV.

Treatment	No. of insects dying without iridescence before day n*1	No. of insects not developing iridescence after day n *1	Percentage of insects developing iridescence *2	Mean incubation period (days)	Mean incubation period (100/days)
vaseline	1	5	89.8	8.75	12.129
lanoline	6	9	79.5	9.77	11.087
soft yellow paraffin	1	9	81.6	8.90	12.479
collodion	32	0	100.0	8.22	12.854
'New-Skin'	5	2	95.6	7.95	13.125
controls	3	3	93.6	8.39	12.352

*1 n is the day of the mean incubation period for that treatment.

*2 Corrected for deaths due to the treatment.

Table 4. Analysis of variance table for mean incubation periods (100/days).

Source	Degrees of freedom	Mean sum of squares	F
Lanoline v. others	1	63.1195	8.1375**
Between others	4	6.3506	1 NS
Error	218	7.7566	
Total	223		

** significant difference ($p < 0.01$).

NS no significant difference.

virus adsorption onto host cells. More than half of the larvae treated with collodion died within a few days, this toxic effect possibly being due to the acetone in which the collodion was dissolved. The coefficient of variation for the incubation periods was found to be high (22.6%), thus again demonstrating the high variability of the larvae in their response to TIV.

There appeared to be less bleeding after the injection in many of the treated larvae, compared with the controls, but as none of the treatments resulted in significantly shorter incubation periods, it was concluded that loss of virus when the insect bleeds is not significant, and no attempts were made to prevent bleeding in the injection procedure.

Experiment 4. Comparison of techniques for the detection of TIV.

Introduction

In many experiments, insects which died without developing iridescence were tested for TIV by making an extract and either centrifuging it to see if an iridescent pellet was formed, or performing a latex test upon it (see Materials and Methods, Detection of TIV). A short experiment was carried out to determine the relative efficiencies of these two techniques.

Methods

Fifty larvae were infected with TIV by injection, and at intervals of 24 h, 10 were selected by random numbers. Each was ground in 2 ml tris HCl + PVP and centrifuged at 600 g for 5 min. One drop of the supernatant was used for a latex test, and the remainder was centrifuged at 7,600 g for 20 min, after which the tube was examined for the presence of an iridescent pellet. On each day 2 control larvae, which had been injected with ISA, were subjected to the same treatment.

Results and Discussion

The results (Table 5) indicate that the latex test is slightly more sensitive than the centrifugation technique for detecting TIV. In many cases the iridescence in the pellet was just a faint streak amongst a lot of grey debris, and was not always easily seen. Latex tests were also carried out on haemolymph withdrawn directly from larvae and on extracts of larvae which had not been subjected to the low-speed centrifugation; many of these resulted in false positives, which did not occur when the larval extract was clarified by centrifugation.

The latex test carried out on the TIV preparation in Experiment 2 resulted in an end-point of 1/2,000; this means that the test detected

Table 5. Numbers of larvae in which TIV infection was detected by the latex test and by centrifugation.

Day	No. tested	No. displaying iridescence	No. TIV-positive by latex test	No. TIV-positive by centrifugation
1	10	0	0	0
2	10	0	3	2
3	10	0	5	4
4	10	0	9	8
5	9	2	9	8

4.80×10^4 LD50 units TIV/ml. In another test the lowest concentration of TIV detected was 4.53×10^4 LD50 units/ml. The tube precipitation test is approximately 10 times less sensitive than the latex test.

The most sensitive technique for detecting TIV is by injection into larvae. If a number of larvae are each injected with 0.005 ml, then it should be possible to detect concentrations of about 2.0×10^2 LD50 units TIV/ml. The latex test is about 100 times less sensitive than this technique, but it has the great advantage that is is much less time-consuming and it was used in the majority of cases when testing for TIV infections in insects which died without developing iridescence. In some experiments the centrifugation technique was employed for this purpose.

Test for a visible reaction between TIV and unsensitised latex particles.

Hukuhara and Hashimoto (1966a) observed that TIV and unsensitised latex particles formed aggregates in aqueous suspension; their formation took several hours, and they gradually increased in size over several days. Decimal dilutions of a TIV suspension were therefore tested against unsensitised latex particles to determine whether any visible aggregation occurs within the time taken to read the latex test, i.e. 40 min. No visible reaction occurred with the unsensitised particles, while the sensitised particles formed visible aggregates with the 10^{-3} dilution of TIV. It would appear, therefore, that any non-specific aggregation of TIV and latex particles is unlikely to interfere with the reading of the latex test.

Experiment 5. The recovery of TIV from aqueous suspensions.

In a number of experiments it was necessary to recover TIV from aqueous suspensions, e.g. when testing larval faeces for the presence of TIV (Experiment 9). A quick test was performed to estimate the percentage of virus recovered from an aqueous suspension by centrifugation. Decimal dilutions of a TIV suspension were prepared, and the 10^{-4} and 10^{-5} dilutions were injected into larvae, 10 larvae being injected with each dilution. One ml of the TIV suspension was then diluted 1/25 with 0.85% NaCl, and centrifuged at 7,600 *g* for 20 min. The pellet was resuspended in 1 ml ISA, decimal dilutions were prepared, and the 10^{-4} and 10^{-5} dilutions were assayed as before.

Table 6. Assay of TIV before and after centrifugation.

	Dilution of TIV	
	10^{-4}	10^{-5}
before centrifugation	7/9	0/10
after centrifugation	6/10	1/10

Each result is expressed as the number of insects developing iridescence, followed by the number injected less any which died without iridescence before the day of the mean incubation period for that dose-group.

The results (Table 6) indicate that this technique did not result in any significant loss of TIV; it was therefore adopted for recovering TIV from aqueous suspensions.

Experiment 6. Assessment of membrane filtration as a technique for the purification of TIV suspensions.

Introduction

In some experiments, e.g. Experiment 15, it was necessary for TIV suspensions to be free from bacterial and fungal contaminants, and membrane filtration was considered as a possible means of achieving this. Tanada and Tanabe (1965) filtered TIV suspensions in distilled water through a 0.3 µm membrane in the final stage of the purification of the virus. The diameter of all reported bacteria is greater than 0.22 µm, therefore a membrane with this pore size should retain all bacteria (Mulvany, 1969).

Preliminary experiments with TIV suspended in ISA suggested that large amounts of the virus were adsorbed onto the membranes. Workers with other viruses have also encountered this problem. Ver et al (1968) found that viruses were adsorbed by membrane filters with pore sizes twice the diameter of the virions, and Cliver (1968) found that enteroviruses were adsorbed significantly at a pore size 285 times greater than the virus diameter. Rao and Labzoffsky (1969) observed strong adsorption of poliovirus onto a 0.45 µm membrane. These investigators diminished this adsorption either by treating the membrane with a protein, which presumably blocks the adsorbing sites, or by suspending the virus in distilled water or a detergent.

Tests were performed to see how adsorption of TIV onto membranes can best be controlled, and to determine whether bacteria and fungi are retained by the membranes.

a) Membrane filtration of TIV in various suspending media.

Methods

Equal quantities of TIV were suspended in 1 ml of each of the following: ISA, deionised water, 0.001% Tween 80, 0.001% Teepol 'XL'. The TIV suspension in ISA was assayed by injecting larvae with decimal dilutions, each dilution being injected into 3 larvae. Each suspension was then filtered through a 0.2 µm membrane and each filtrate was assayed by preparing decimal dilutions with ISA and injecting each dilution into 3 larvae.

Results and Discussion

The results (Table 7) indicate that virtually all the TIV suspended in ISA was adsorbed by the membrane. This adsorption was diminished by suspending the virus in deionised water or a detergent, but only about 0.1% of the virus was recovered, so there was still significant adsorption. It appears, therefore, that ions play an important role in the adsorption process. Rao and Labzoffsky (1969) found that the adsorption of poliovirus on to membrane filters was enhanced by the presence of calcium ions. Some workers (e.g. Cliver, 1968) have treated membranes with protein to control the adsorption of viruses, so tests were done to see if this procedure will control the adsorption of TIV.

b) Treatment of membranes with serum, and tests for the retention of bacteria and fungi by the membranes.

Methods

A TIV suspension in distilled, deionised water was prepared and assayed by the response time method. It was then filtered successively

Table 7. Assay of TIV suspensions before and after filtration through membrane filters.

Dilution	Before filtration	After filtration, TIV suspended in:			
		ISA	Deionised water	Tween 80	Teepol 'XL'
10^{0}	3/3	0/3	3/3	3/3	3/3
10^{-1}	3/3	0/3	2/3	2/3	1/3
10^{-2}	3/3	0/3	1/3	1/3	2/3
10^{-3}	2/3	0/3	0/3	2/3	0/3
10^{-4}	3/3	0/3	0/3	0/3	0/3
10^{-5}	1/3	0/3	0/3	0/3	0/3
10^{-6}	1/3	0/3	0/2	0/1	0/3

Each result is expressed as the number of insects developing iridescence, followed by the number injected less any which died without iridescence before the day of the mean incubation period for that dose-group.

The dotted lines indicate approximate positions of the LD50s.

through 2.0, 1.2, 0.8, 0.45 and 0.2 μm membranes, previously treated with protein by filtering through each approximately 1 ml sterile rabbit serum. After each filtration, the suspension was assayed by the response time method, and tested for the presence of bacteria and fungi by spreading 0.1 ml aliquots on 6 plates each of nutrient agar and malt extract agar. Two plates of each medium were incubated at each of the following temperatures: 5^{o}, 20^{o}, 37^{o}.

Results and Discussion

The results of the response time assays were highly variable, and in many cases the values extended beyond those of the standard curve (Fig. 12) so that no estimate could be made. All the filtrates, however, proved to be highly infective, and there appeared to be little or no virus loss. The estimate of the TIV titre before filtration was 6.0×10^{7} LD50 units/ml, and that for the final filtrate was 2.2×10^{8} LD50 units/ml.

The tests for the presence of bacteria and fungi in the filtrates (Table 8) indicated that the 0.45 μm membrane effectively held back bacteria, while the 1.2 μm membrane effectively held back fungal spores. The numbers of bacterial and mould colonies growing on the nutrient agar plates inoculated from the 0.45 μm and 0.2 μm filtrates were similar to those growing on control plates across which a sterile glass spreader had been streaked.

It has thus been demonstrated that filtration of a TIV suspension through membrane filters with progressively smaller pore diameters, finishing with a 0.2 μm membrane, should render it free from bacteria and fungi, and that loss of virus due to adsorption onto the membranes should be negligible if the membranes are coated with serum and the virus is suspended in demineralised water.

Table 8. Results of tests for the presence of bacteria and fungi in filtrates of a TIV suspension.

	Nutrient agar			Malt extract agar		
	5°	20°	37°	5°	20°	37°
before filtration	uncountable	uncountable	uncountable	uncountable	uncountable	0
2.0 µm filtrate	uncountable	uncountable	uncountable	uncountable	uncountable	0
1.2 µm filtrate	uncountable	uncountable	uncountable	0	0	0
0.8 µm filtrate	uncountable	uncountable	uncountable	0	0	0
0.45 µm filtrate	1 mould	0	0.5 bacteria	0	0	0
0.2 µm filtrate	0.5 mould	0	0	0	0	0

Each result is the mean value from 2 plates, each spread with 0.1 ml TIV suspension.

The plates incubated at 20° and 37° were examined after 2 days, and those at 5° after 7 days.

Experiment 7. Determination of larval instar by measurement of spiracle diameter.

Introduction

In some experiments it was important to know the larval instar. Size and weight are unreliable guides to larval instar as there may be much variation; some larvae may be smaller than others in the preceding instar. The diameter of the spiracles remains fairly constant throughout an instar, and then approximately doubles or trebles in diameter in the next instar, so spiracle diameter is an excellent guide to larval instar.

Methods

Larvae from 10 different progeny groups were reared at 20° and the diameter of their spiracles was measured daily, from hatching to pupation, using a stereoscopic microscope fitted with a micrometer eyepiece. The experiment was commenced with a total of 175 larvae, and 21 survived to pupation.

Results and Discussion

The spiracle diameters of the larval instars are given in Table 9.

Table 9. Spiracle diameters of larval instars of T. oleracea.

Larval instar	Spiracle diameter (mm)
I	0.04
II	0.12
III	0.33
IV	0.62

There was little variation between the spiracle diameters of different larvae in any one instar. The development of the insects at 20° is depicted in Fig. 13, which can be used to estimate the proportions of larvae in the different instars on any day after hatching. There was high mortality among the larvae, especially during the early instars, so that the number of larvae which reached the fourth instar was small compared with the initial number.

There were differences in the rate of development between different progeny groups (Table 10), and these differences were significant in the early instars (Table 11). The lack of significance in the later instars may be due to the fact that there were few larvae surviving. There were also big differences in the rate of development between individuals within the progeny groups.

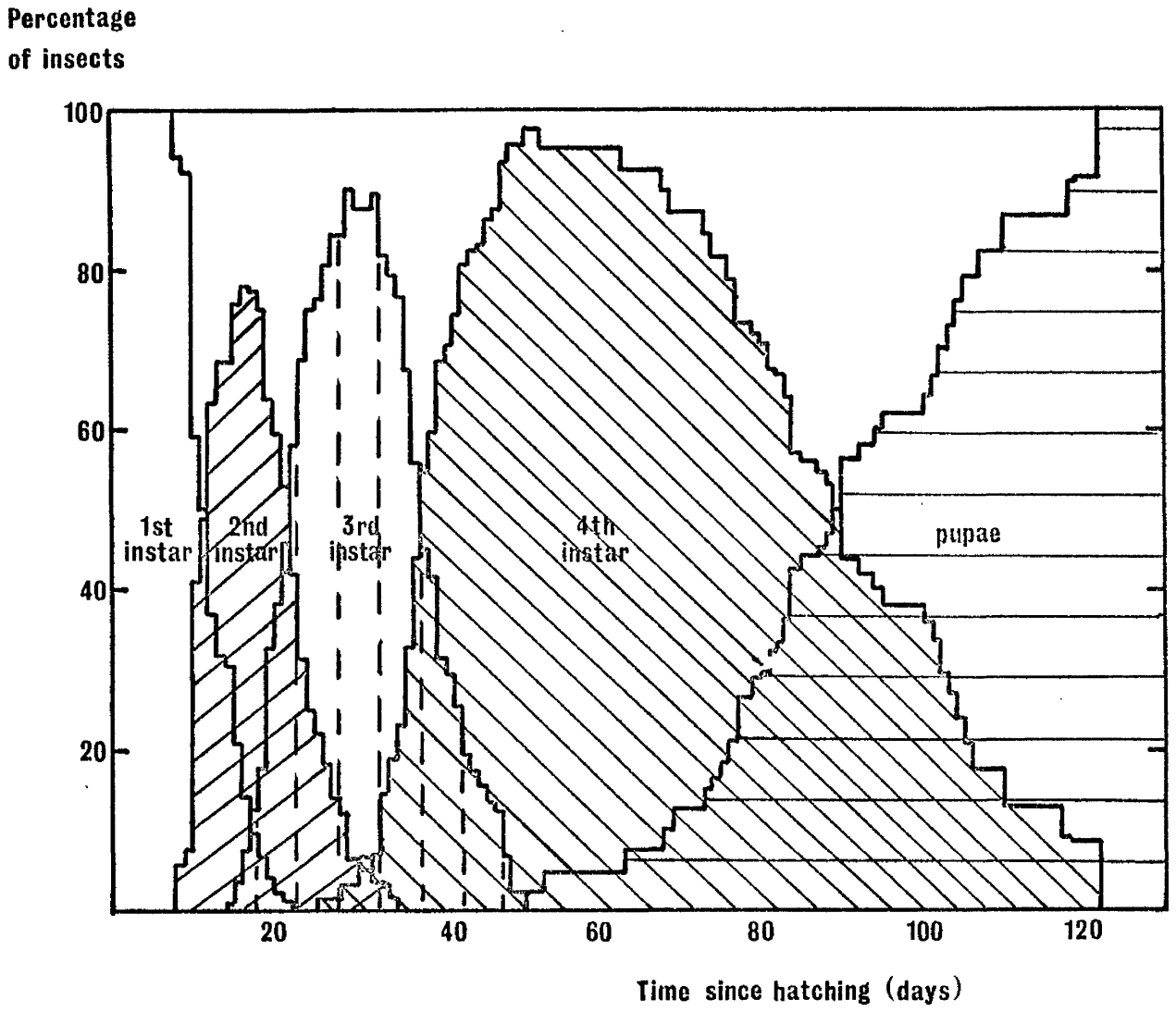


Fig.13. Development of T.oleracea at 20°.

Table 10. Rates of development of different progeny groups at 20°.

Progeny group	Larval instar			Pupa
	II	III	IV	
1	14	23	41	54
2	17	34	69.5	120
3	17	29.5	64	111
4	18	32	62.5	123
5	15	25	47.5	88
6	16	29.5	57.5	108
7	13.5	30	76.5	- ≠
8	25	31.5	- ≠	- ≠
9	23.5	41	58	69
10	20	30	54.5	78

Each result is the mean day on which the majority of the progeny group were in that stadium.

≠ All insects dead.

Table 11. Results of χ^2 tests upon the data in Table 10.

Time since hatching (days)	No. of progeny groups compared ≠	Instars	χ^2
14	9	1, 2	69.87***
21	7	2, 3	12.72*
28	5	2, 3	3.16 NS
35	5	3, 4	4.72 NS

≠ Progeny groups with few surviving larvae were not included.

*** Significant difference ($p < 0.001$)

* Significant difference ($p < 0.05$)

NS No significant difference.

B. MODE OF TRANSMISSION OF TIV: SOURCE OF INFECTION

Introduction

If leatherjackets pick up a TIV infection from their environment, the virus must come ultimately from other infected insects. The source of infection could be the infected insects themselves or virus in their excreta.

Experiment 8. Demonstration of the transmission of TIV

Introduction

There are few reports in the literature of observations on the transmission of a pathogen from one individual insect to another. Jaques (1962) demonstrated the transmission of the NPV of *Trichoplusia ni* from infected to healthy larvae, but Van der Lann and Wassink (1969) found that hardly any healthy larvae of the Mediterranean flour moth, *Ephestia kühniella* (Zeller), or *Pieris brassicae* became infected when they were confined with larvae of the same species infected with *Bacillus thuringiensis*. In order to test whether TIV can be transmitted from one larva to another, healthy and TIV-infected leatherjackets were confined together in petri dishes.

Results

a) Confinement of healthy fourth-instar larvae with infected fourth-instar larvae

Thirty petri dishes were prepared, each containing 1 TIV-infected fourth-instar larva (the primary larva); from 1 - 5 healthy fourth-instar larvae (the secondary larvae) were placed in each dish. In 18 of the dishes some or all of the secondary larvae became infected with TIV, whereas in control dishes, containing only healthy larvae, no insects

became infected. Some correlation was found between the period elapsing up to the death of the primary larvae and the period elapsing before the appearance of TIV symptoms in the secondary larvae ($r = 0.695$). 48.3% of the variation in the time for appearance of symptoms in the secondary larvae can be accounted for by the time elapsing before the death of the primary larvae.

However, it is possible that some event occurs before the death of a primary larva and that the correlation is with the period elapsing before this event rather than with the time lapse up to death. To test this possibility, the following experiment was performed. One TIV-infected (primary) larva and one healthy (secondary) larva were placed in each of 77 petri dishes. On each subsequent day the primary larvae were removed from 2 dishes selected at random, and the secondary larvae were observed for the development of iridescence. Six of the 77 secondary larvae became infected with TIV; in all 6 cases the primary larva had died, and had been partially or completely ingested. In no case where the primary larva was removed from the dish did the secondary larva become infected, which suggests that the correlation of time for appearance of iridescence in secondary larvae is with the time to death of the primary larvae, i.e. that the infected cadaver was the source of infection.

There were 11 other cases where the primary larva died and was partially or completely ingested by the secondary larva; none of these developed iridescence, but they could have been infected with TIV at a low level, as no further tests for TIV were carried out on them.

Most infected (primary) larvae appeared to remain quite active and

pugnacious until a short time before they died and a number of secondary larvae were ingested by primary larvae as well as vice versa.

In order to test the converse of the previous experiment, 1 primary and 10 secondary larvae were placed in each of 5 petri dishes. Dishes containing 11 non-infected larvae were prepared as controls. At various time intervals 1 secondary larva was removed from each dish and transferred to an individual dish. The time of death of each primary larva was noted.

Thirteen secondary larvae were removed from the test dishes before the primary larva had died; none of these developed iridescence. Thirty-seven were removed after the primary larva had died; 6 of these subsequently developed iridescence, and in each of these cases the primary larva had been partly or completely ingested. None of the control larvae developed iridescence.

The final experiment of this series was designed to determine the possible effect of population density on the proportion of larvae becoming infected. One TIV-infected larva was placed in each of 100 petri dishes. From 0 - 9 healthy larvae were added to each dish, each population density (from 1 - 10 per dish) being replicated 10 times. There was also 1 control dish at each population density, containing only healthy larvae.

The results (Table 12) indicate that there is little correlation between the proportion of larvae becoming infected and the population density. Only 5.5% of the variation in the proportion of larvae becoming infected can be explained by variations in population density.

Table 12. Numbers of larvae becoming infected at different population densities.

Total no. larvae/dish	No. becoming infected	Number becoming infected / Total no. secondary larvae	Percentage becoming infected
2	10 x 0	0/10	0.0
3	8 x 0; 2 x 1	2/20	10.0
4	10 x 0	0/30	0.0
5	8 x 0; 1 x 1; 1 x 2	3/40	7.5
6	7 x 0; 2 x 1; 1 x 4	6/50	12.0
7	8 x 0; 1 x 1; 1 x 4	5/60	8.3
8	6 x 0; 3 x 1; 1 x 2	5/70	7.1
9	2 x 0; 3 x 1; 3 x 2; 2 x 3	15/80	18.8
10	5 x 0; 2 x 1; 2 x 2; 1 x 5	11/90	12.2

Regression coefficient (b) = 1.50.

During this experiment, each primary larva was carefully observed post mortem, to determine whether or not it was ingested, completely or partly, by the secondary larvae. In some cases it was difficult to ascertain whether or not it had been partly ingested, as the cadaver began to decompose and disintegrate. In a few cases the secondary larvae were observed in the act of feeding on the cadaver of the primary larva. The results of the observations are summarised in Table 13, and suggest that ingestion of the primary larva was the main mode of infection and that the probability of infection of the secondary larvae was in direct proportion to the amount of infective material ingested.

There was some evidence that a higher population density contributed towards a more rapid death of the primary larvae (Table 14).

25.5% of the variation in time to death of the primary larvae can be attributed to variations in population density. This may be due to a greater chance of being attacked, or a greater accumulation of toxic excretory products at higher population densities.

Combining all the results from the separate parts of this experiment, fairly good correlation ($r = 0.848$) was found between the time for appearance of iridescence in the secondary larvae, and the time taken for the primary larvae to die (Fig. 14).

b) Confinement of healthy first-instar larvae with infected fourth-instar larvae.

As a preliminary experiment, 8 petri dishes, each containing 1 TIV-infected fourth-instar and 20 healthy first-instar larvae were prepared. Many of the latter died from desiccation within a few days, as

Table 13. Effect of ingestion of primary larva upon probability of infection in secondary larvae.

Fate of the primary larva	No. of dishes	No. of dishes where secondary larvae became infected	Percentage of dishes where secondary larvae became infected
Not observed to be ingested	39	2	5.1
Uncertain whether it was partly ingested	10	1	10.0
Partly ingested	24	9	37.5
Completely ingested	17	14	82.4

Table 14. Times to death of primary larvae at different population densities.

Population density (No. of larvae per dish)	Mean time to death of primary larva (days)
1	21.6 (3.6)
2	20.7 (4.1)
3	17.8 (4.9)
4	18.7 (4.2)
5	15.2 (6.7)
6	13.8 (5.3)
7	14.1 (4.8)
8	15.7 (7.6)
9	12.0 (5.3)
10	13.5 (5.2)

Standard deviations are indicated in brackets.

Regression coefficient (b) = -1.08.

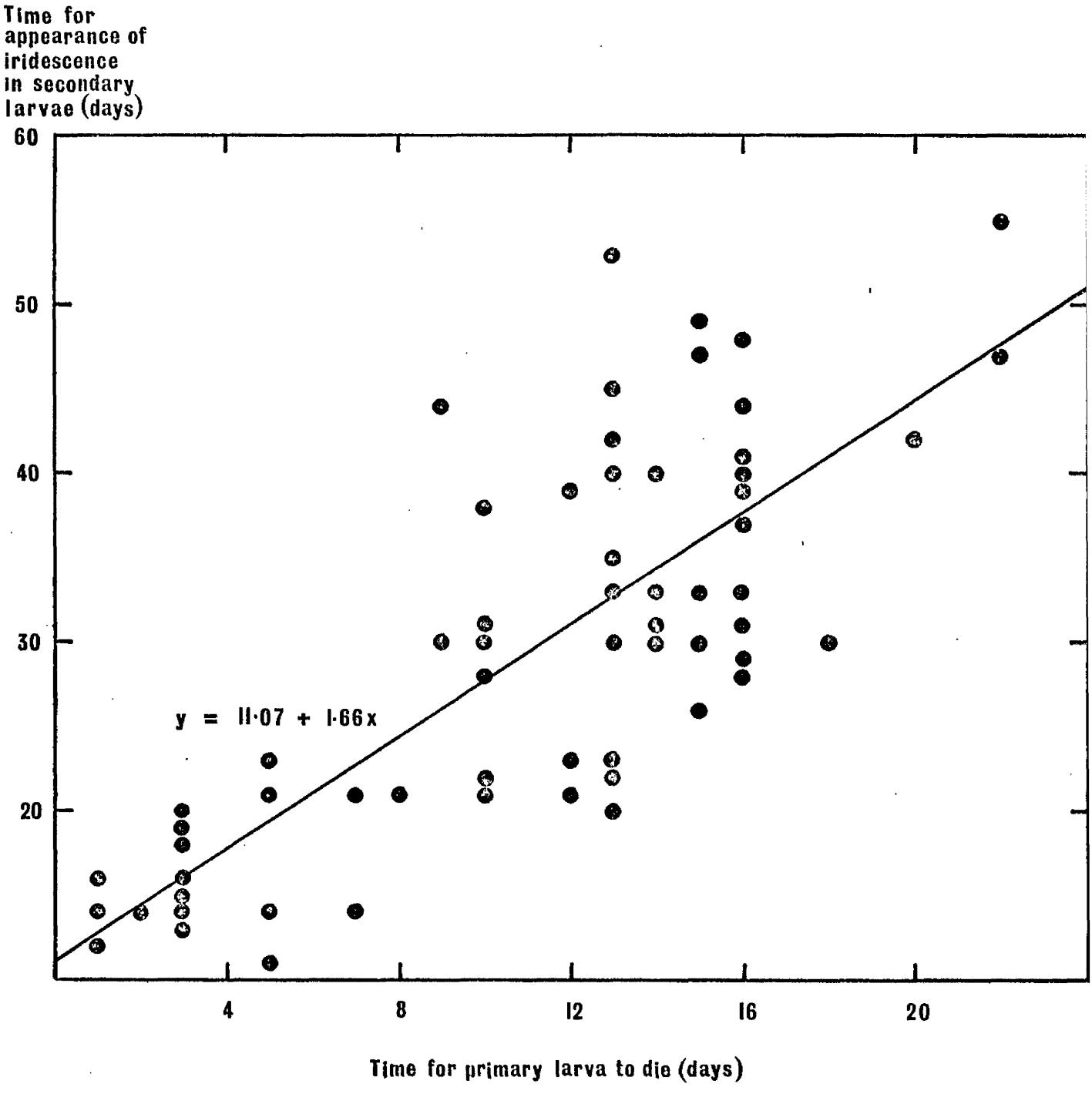


Fig.14. Correlation between incubation period in secondary larvae and time for primary larvae to die.

they crawled from the moist filter papers to the sides and lids of the dishes. Most of the survivors died from TIV-infections in later instars. None of the secondary larvae developed TIV symptoms until after the primary larvae had died. Two of the secondary larvae pupated, but no adults emerged.

In the main experiment, 30 dishes were prepared as before along with 30 control dishes each containing 1 healthy fourth-instar and 20 healthy first-instar larvae. In the dishes containing an infected primary larva 18 (3.0%) of the secondary larvae survived to pupate, whereas in the control dishes 33 (5.5%) of the secondary larvae pupated. The χ^2 test indicates a significant difference ($p<0.05$) between these 2 results. Large numbers of secondary larvae in both sets of dishes died in the first instar due to desiccation, and many secondary larvae in the dishes with infected primary larvae contracted a fatal TIV infection after the primary larva had died.

Discussion

The results suggest that the infected cadavers were the source of infection for the healthy larvae, and that infection was by ingestion of the cadavers. It was shown that death of the primary larva was necessary before any secondary larvae became infected. It would appear, therefore, that the secondary larvae did not contract the disease by contact with either the primary larvae while still alive, as for example during fighting, or with any TIV excreted by the primary larvae.

Ahmed (1968) observed cannibalism on virus-diseased T. paludosa larvae by healthy larvae, and vice versa. Cannibalism on diseased larvae increased the rate of infection among the cannibals. He also observed

that some TIV-infected larvae may survive for up to 13 weeks and some NPV-infected larvae for up to 14 weeks. Successive generations of larvae could therefore be infected by diseased larvae which had survived from the previous generation as demonstrated in the present experiment (8b).

In this experiment, population density was found to have little effect on the proportion of larvae becoming infected. There are, however, a number of reports of increased incidence of disease among crowded populations of insects, e.g. the NPV of Colias eurytheme (Thompson and Steinhaus, 1950), polyhedroses of tent caterpillars, Malacosoma spp., (Clark, 1958), the NPV of Phryganidia californica (Packard) (Martignoni and Schmid, 1961), and a non-inclusion virus of the European red mite, Panonychus ulmi (Koch), (Putman, 1970). Linley and Nielsen (1968a) and Woodard and Chapman (1968) found that crowding Aedes taeniorhynchus larvae in the presence of NIV did not significantly affect the rate of infection, but Matta and Lowe (1970) reported that mortality was dependent upon the population density of the larvae.

Newbold, J.W. (personal communication) has observed a field with a very high population density of leatherjackets (4.9 million/hectare) with 75% of them infected with TIV, and Stewart (1969) found parasites and disease in tipulids most often in dense populations, although dense populations were frequently free of parasites and disease.

In the present experiment the larvae were not placed at the varying population densities until most of the larval stage was completed, i.e. when they were in the fourth-instar. By the time the primary larvae died, most of the secondary larvae were nearing pupation, and some had pupated.

In the natural situation, however, the larvae may be exposed to infected individuals throughout their whole life, with a possible increase in number of infected individuals with time, so that under natural conditions it may be that the population density affects the proportion of larvae becoming infected.

The results indicate that the presence of TIV-infected individuals in a field population of leatherjackets in the early instars might effect a significant reduction in the population size.

Experiment 9. The excretion of TIV by infected leatherjackets.

Introduction

Smirnoff (1961) examined the faeces of NPV-infected larvae of the sawfly, Neodiprion swainei, and was unable to find polyhedra, although aqueous extracts were infective. Jaques (1962) found that the excreta of Trichoplusia ni larvae infected with NPV were not highly infective, but Bailey and Gibbs (1964), working with acute bee paralysis virus in the honey bee, Apis mellifera (Linnaeus), found that the virus remained infective for long periods of time in dry faeces, which could thus provide a source of infection for other bees.

The excreta of TIV-infected leatherjackets were examined for the presence of TIV and as a possible source of infection for other leatherjackets.

a) Preliminary experiments

Ten larvae were infected with TIV by injection. Their faeces were collected at various times, and shaken with distilled water. The suspension was centrifuged at 600 g for 5 min, then the supernatant was centrifuged at 7,600 g for 20 min. The pellet was subjected to a similar cycle of low-/high-speed centrifugation, and the final pellet was suspended in 0.5 ml ISA. This was tested for the presence of TIV by injection into 5 larvae. The results of 2 such experiments (Fig. 15) show that virus was present in the faeces of larvae which had been injected with TIV, with 3 possible peaks: one at days 1 - 2, one at days 4 - 7, and one around day 14.

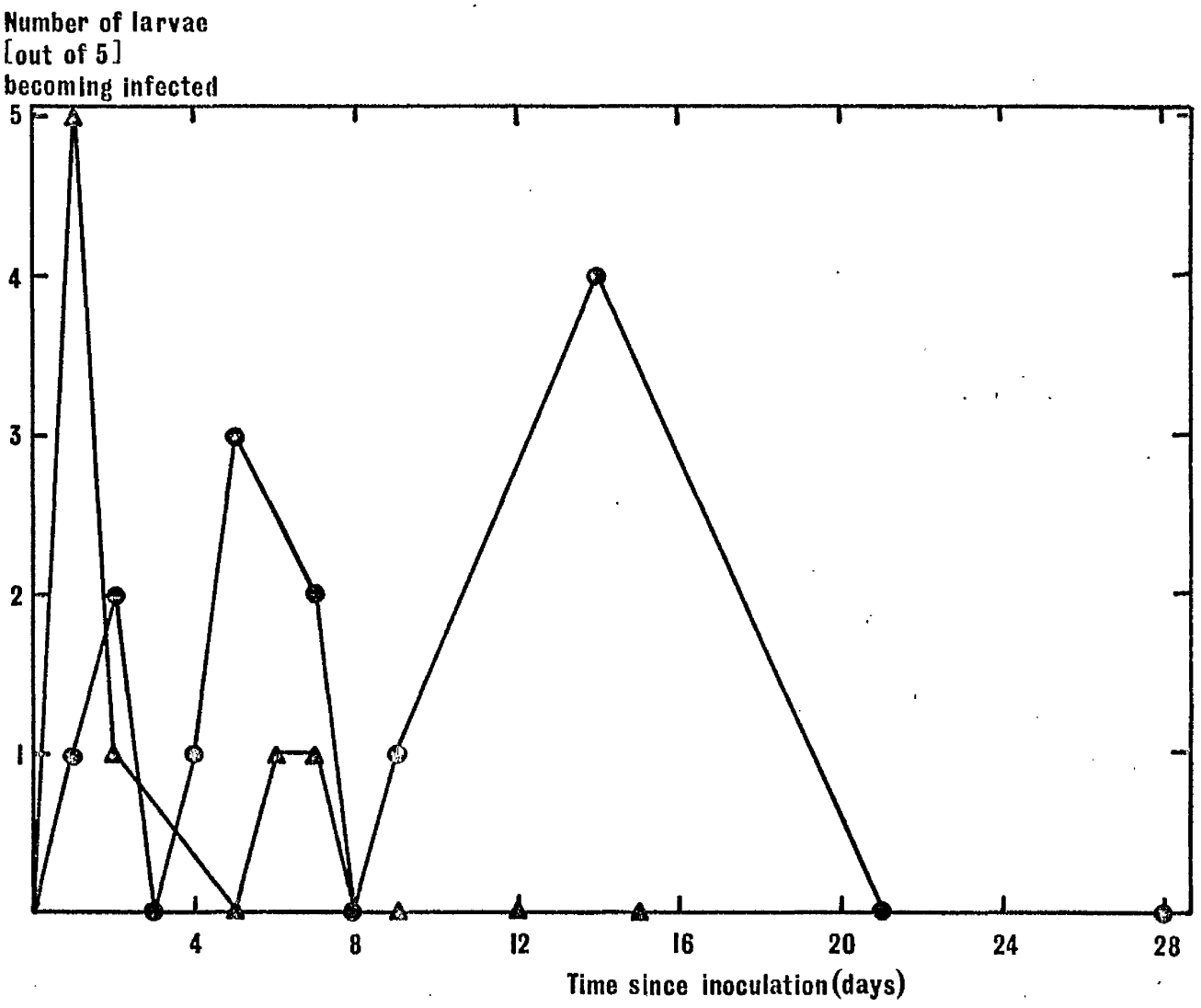


Fig.15. TIV detected in the faeces of infected larvae.

Results of 2 experiments

b) Main experiment

The main experiment was similar to the preliminary experiments, but larger numbers of insects were involved. Fifty larvae were each injected with 1.3×10^5 LD50 units TIV, and their faeces were collected daily. TIV in the faeces was assayed by the response time method, 20 larvae being injected with each preparation. Latex tests were also performed on the preparations, but the levels of TIV were too low to be detected by this method.

The results (Fig. 16) were similar to those obtained in the preliminary experiments, with quite large amounts of virus being excreted during the first few days after injection with TIV, and another peak about 2 weeks later. Possibly the first peak is some of the injected virus, excreted following phagocytosis in the haemocoel and transfer to the gut, whereas the second peak probably consists of virus which has been synthesised in the insect.

The quantity of food ingested, and hence the quantity of faeces excreted, began to decrease gradually from approximately day 20, and it was around this time that the larvae began to die from the disease.

Although the quantities of virus excreted would appear to be quite high, e.g. 32.5 LD50 units per insect on day 16, it must be remembered that these LD50 units are measured by injection, and that the LD50 by feeding is vastly greater (Experiment 13). It would be unlikely that the amounts of virus excreted by these larvae would be sufficient to infect other larvae *per os*.

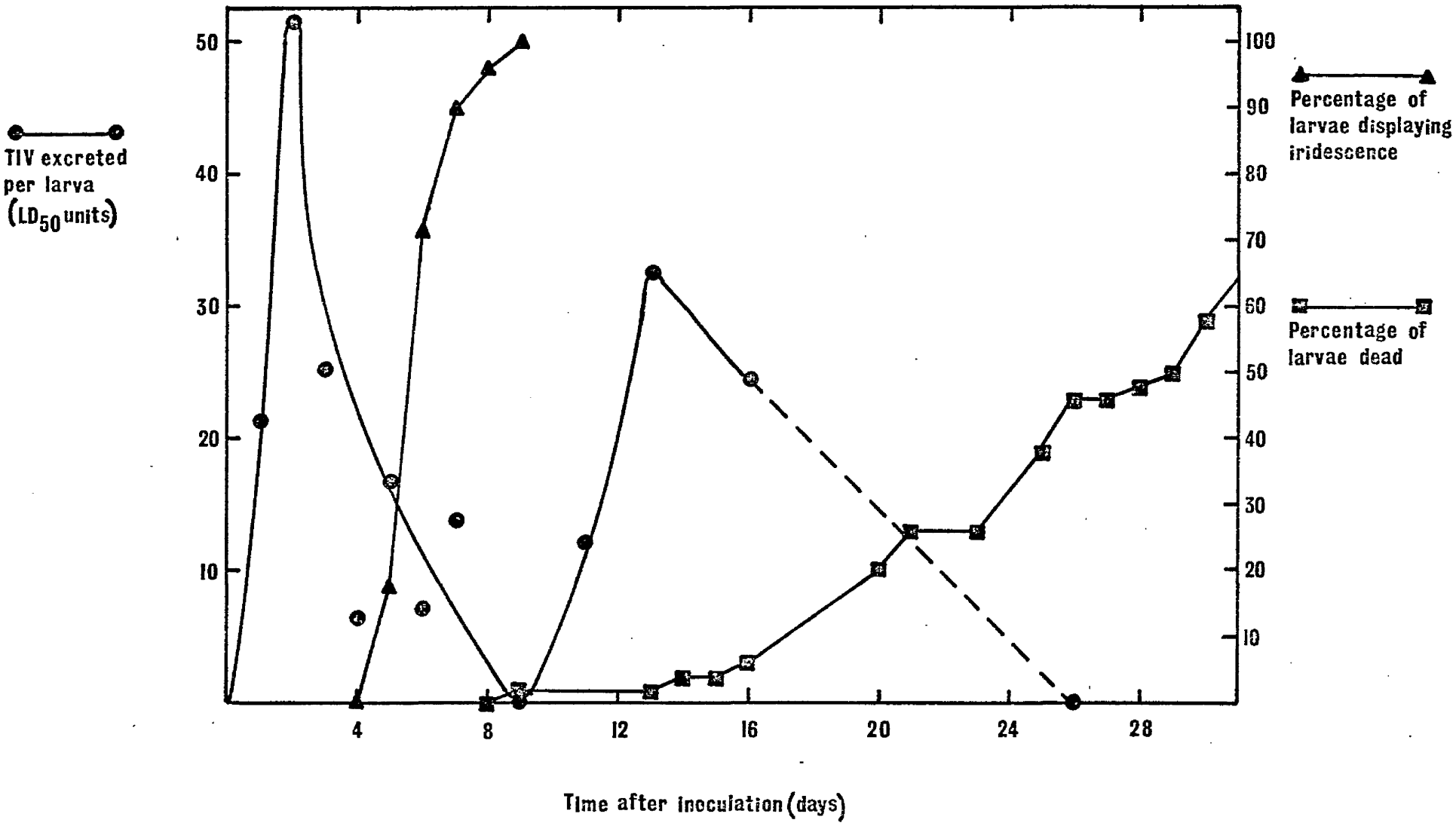


Fig.16. The excretion of TIV by infected larvae.

Experiment 10. To determine whether excreta from infected larvae can serve as a source of infection

Introduction

Bailey and Gibbs (1964) found that the faeces of infected bees could serve as a source of infection for acute bee paralysis virus, and Putman (1970) suggested that the mite Panonychus ulmi becomes infected with a non-inclusion virus while feeding on the excreta or oral secretions of infected mites. Stevenson (1959), however, found no evidence that faecal contamination was important in the epizootiology of Serratia marcescens infections of the desert locust, Schistocerca gregaria (Forskål), and Linley and Nielsen (1968b) found that when they placed healthy Aedes taeniorhynchus larvae in pans which had contained MIV-diseased larvae, none of them became infected.

It was shown (Experiment 9) that the faeces of TIV-infected insects contain virus, but in amounts which are unlikely to infect other larvae. Healthy larvae were confined with the faeces of TIV-infected larvae in order to check whether this is a likely source of infection.

a) First-instar larvae

In one experiment 20 first-instar larvae were placed in each of 9 petri dishes which had contained 1 TIV-infected larva for 13 days. In another experiment, 10 first-instar larvae were placed in each of 2 dishes along with the faeces collected from 10 TIV-infected fourth-instar larvae.

No evidence of TIV infection appeared in any of the larvae in these experiments.

b) Fourth-instar larvae

(i) From 1 - 5 infected larvae were kept in each of 10 dishes for 8 days. The infected larvae were then removed and replaced by 1 fourth-instar larva in each dish.

(ii) Faeces of TIV-infected larvae were collected and placed in 25 petri dishes. Each dish received the faeces of 10 larvae, along with 1 fourth-instar larva.

None of the larvae in these experiments, and none of the control larvae, developed evidence of TIV infection. However, TIV was detected by a latex test in an apparently normal female crane-fly which emerged following the pupation of one of the 25 larvae confined with large quantities of contaminated faeces. It seems probable that a sub-lethal infection was contracted from the faeces. High concentrations of contaminated faeces, to which this insect was exposed, are unlikely to be found in nature, hence it is improbable that leatherjackets in their normal environment become infected with TIV from the faeces of infected insects.

Experiment 11. To determine whether the cadavers of infected larvae can serve as a source of infection

Introduction

The experiments of Stevenson (1959) indicated that cannibalism plays a part in the epizootiology of Serratia marcescens infections of Schistocerca gregaria, and Linley and Nielsen (1968b), suggested that MIV infections of Aedes taeniorhynchus occur by healthy larvae feeding on diseased cadavers. The fact that tipulid larvae are cannibalistic has been noted (Laughlin, 1958; Freeman, 1966), and Ahmed (1968) found that T. paludosa larvae became infected with TIV when they were released into pots of sand containing the dried cadavers of TIV-infected leatherjackets. It was suggested in Experiment 8 that those larvae that became infected with TIV did so by ingesting diseased larvae. The following tests confirmed that TIV can be transmitted in this way.

a) First- and second-instar larvae

Petri dishes were prepared, each containing one cadaver of a TIV-infected fourth-instar larva and 10 healthy first- or second-instar larvae. Three separate tests were conducted, the results of which are given in Table 15.

Large numbers of larvae became infected in all 4 instars, but 34.8% of the dishes containing second-instar larvae at the outset did not develop TIV infections in their larvae, compared with 4.8% of the dishes which contained first-instar larvae; the χ^2 test indicates that this difference is significant ($p<0.05$).

Table 15. Numbers of dishes in which larvae developed TIV symptoms

	Larval instar at commencement of test	
	First-instar	Second-instar
Test (i)	4/5	1/5
(ii)	10/10	7/10
(iii)	6/6	7/8
Totals	20/21 (95.2%)	15/23 (65.2%)

The results are given as the number of dishes in which TIV symptoms appeared in the larvae/total number of dishes.

χ^2, 1 d.f., = 4.38.

Table 16. Numbers of fourth-instar larvae becoming infected/total number

Test (i)	8/39
(ii)	12/54
Total	20/93 (21.5%)

b) Fourth-instar larvae

Petri dishes, each containing 1 cadaver of a TIV-infected fourth-instar larva and 1 healthy fourth-instar larva, were prepared. Two replicate tests were conducted, the results of which are given in Table 16.

Of the 20 insects which became infected, 5 developed TIV symptoms in the larva, and 2 emerged as adults with TIV symptoms (see Experiment 19); the other infections were detected in larvae, pupae and adults by the latex test.

Discussion

The results suggest that the ingestion of a TIV-infected cadaver by a healthy larva may result in that larva becoming infected, and that the larvae are most susceptible in the first instar, resistance increasing with age. Successive generations of leatherjackets might become infected in this way if diseased individuals from the previous generation are still present in the soil.

A massive dose of TIV is required if leatherjackets are to become infected per os (Experiments 13 and 14). The most probable way that a large enough dose can be ingested is by feeding on a diseased insect, of which approximately 25% of the dry weight is TIV (Williams and Smith, 1957 It has been observed in the field that leatherjackets infected with TIV tend to rise to the soil surface (Ahmed, 1968: Newbold, J.W., personal communication), and during the present work most larvae in an advanced stage of the disease crawled from beneath the filter paper before they died. On the soil surface they may be discovered more easily by other larvae feeding at the surface, although they will also be more easily

discovered by predators such as birds. Rivers (1966b) pointed out that cannibalism is a mode of transmission involving minimum loss of viability to the pathogen.

Experiment 12. Determination of the extent to which TIV-infected leatherjackets contaminate their environment with virus.

Introduction

There are few reports of insect viruses being recovered from the soil. Thompson and Steinhaus (1950) found in the soil of alfalfa fields a NPV which was infectious for Colias eurytheme. Jaques (1967) treated plots with the NPV of Trichoplusia ni, and 5 years later recovered approximately 25% of the infective virus, but Linley and Nielsen (1968b) could not recover infective MIV from soil 1 day after the virus was applied to it.

Methods

Leatherjackets were kept in small pots (65 mm diameter) of sand, with 10 insects per pot. One pot contained larvae which had been infected with TIV by injection, a second contained ISA-injected larvae, and a third pot contained no larvae. Any dead larvae found on the surface of the sand were replaced with living larvae, either TIV-infected or not, as appropriate, in an attempt to keep the numbers of larvae in the pots constant.

After 14 days all the larvae were removed from the pots; there were 5 TIV-infected and 6 non-infected larvae remaining. The others presumably had either been ingested or had died and decomposed. A sample of sand was taken from each pot, and the dry weight was measured. The remainder was weighed and shaken with 100 ml 0.85% NaCl, and a 25 ml aliquot of the suspension was subjected to two cycles of low-/high-speed (600 g for 5 min/7,600 g for 20 min) centrifugation. The final pellet was suspended in 1 ml ISA, and this was tested for the presence of TIV by injecting larvae with decimal dilutions. Each dilution was injected into 3 larvae.

Results and Discussion

TIV was found in the sand which had contained TIV-infected larvae, the LD50 of the preparation being between 10^{-3} and 10^{-4} (Table 17).

The dry weight of the sand which was shaken with saline was calculated as 140 g. If the LD50 of the extract is taken as $10^{-3.5}$, then this means that the sand contained approximately 2.4×10^{4} LD50 units/g dry weight. It is unlikely that all of this TIV could be accounted for by virus in the insects' excreta (Experiment 9); some of it is probably also derived from decomposed cadavers. Although this represents a considerable amount of virus material, it is unlikely to be very significant as a source of TIV for infection *per os*. Even if each larva ingested 1.0 g (dry weight) sand, the expected proportion of larvae becoming infected would be very low.

No TIV was detected in the sand containing ISA-injected larvae or in the sand containing no larvae.

Table 17. Numbers of larvae becoming infected after injection with dilutions of a suspension prepared from sand which had contained TIV-infected leatherjackets.

Dilution	No. of larvae becoming infected	Percentage of larvae becoming infected
10^{0}	3	100
10^{-1}	3	100
10^{-2}	3	100
10^{-3}	3	100
10^{-4}	1	33
10^{-5}	0	0

C. MODE OF TRANSMISSION OF TIV: ROUTE OF INFECTION

Introduction

Smith and Williams (1958) stated that it is not known how transmission of TIV occurs, but presumed it to be by ingestion of the virus. There are several possible routes whereby TIV might enter leatherjackets: a) *per os*; b) directly into the haemocoel via cuts in the skin or via parasites of the leatherjacket; c) via the spiracles; d) infection of the embryo; e) vertical transmission via the adults, i.e. transmission from one generation to the next via the egg or the sperm. The following experiments were designed to study these possibilities.

Experiment 13. A comparison of different inoculation routes.

Introduction

It has been established that TIV has a very low infectivity when taken orally by leatherjackets, but that injection of a relatively small dose into the haemocoel will result in infection (Rivers, 1966a). In nature TIV could possibly enter the haemocoel directly through wounds during fighting, or it could be introduced by a parasite. There are several reports of the transmission of insect pathogens by parasites, e.g. the NPV of *Colias eurytheme* can be transmitted on the ovipositor of *Apanteles medicaginis* (Muesebeck) (Thompson and Steinhaus, 1950); the NPV of the lawn armyworm, *Spodoptera mauritia acronyctoides* (Guenee) can be transmitted on the ovipositor of *Apanteles marginiventris* (Cresson) (Laigo and Tamashiro, 1966); and the polyhedrosis viruses of sawflies can be transmitted by parasites (Bird, 1961).

Leatherjackets are parasitised by the insect Siphona geniculata (Rennie, 1912) and the nematode, Mermis albicans (Ahmed, 1968). S. geniculata adults do not insert the ovipositor into the host, but lay their eggs on the surface of the insect, so it is unlikely that they are important as vectors of TIV; there are no reports of parasitism by nematodes being associated with TIV infection. (Stewart, K.M., personal communication) found tachinid larvae, nematodes and TIV parasitising leatherjackets, but he never found a larva parasitised by both TIV and a tachinid or nematode at the same time. Rivers (1966b) has suggested that, as TIV can be readily transmitted to other insect orders, other soil-dwelling organisms may be involved in the transmission of TIV, but there is no evidence for this. He also noted the habit of some insects, including leatherjackets, of biting each other, and pointed out that this is a mode of transmission involving minimum loss of viability to the pathogen.

Leatherjackets were inoculated with TIV by the following 4 techniques, and their efficiency was compared: feeding, injection into the haemocoel, dropping virus onto damaged skin and dropping virus onto undamaged skin.

Methods

The inoculation techniques were as follows:

a) Feeding. Each larva was placed in a dry petri dish and left for 4 h to desiccate, then 0.005 ml TIV suspension was placed in each dish. Most of the larvae drank from the drop. They were left for a further 2 h, then each was provided with a filter paper, grass and SVS.

b) Injection. Each larva was injected with 0.005 ml TIV suspension.

c) Via cuts in skin. Each larva was rubbed on sand-paper until it began to bleed, then 0.005 ml TIV suspension was dropped onto its surface.

d) Via undamaged skin. 0.005 ml TIV suspension was dropped onto the surface of each larva.

These inoculation techniques were carried out on larvae of 4 different ages. In test (i) most larvae were in the second instar, in test (ii) most were in the third instar, and in tests (iii) and (iv) all were in the fourth instar. Control groups received similar treatments, but with ISA substituted for the TIV suspension.

Results

The results are summarised in Table 18. The same numbers of larvae were used for the controls; none of these became infected with TIV. Similar numbers of controls and treated larvae died from the trauma of the treatments and from unknown causes.

The order of efficiency for the inoculation routes was:

1. Injection.
2. Via cuts in the skin.
3. Feeding.
4. Via undamaged skin.

The data for incubation periods and times to death were subjected to analysis of variance (Tables 19 and 20), and significant differences, especially in incubation period, were found between treatments.

There was a tendency for TIV symptoms to appear earlier, and for death to occur more rapidly, in larvae inoculated by a more efficient route (Figs. 17 and 18).

Table 18. Results of inoculating leatherjackets with TIV by different inoculation routes.

Test Number	Mean larval age (days)	Inoculation route	No. of larvae inoculated	No. of larvae becoming infected with TIV	Corrected percentage infection*1	Mean incubation period (days)*2	Mean survival time (days)*2
(i)	17	Feeding	50	1	3.4	11.0	20.0
		Injection	Not done *3	-	-	-	-
		Via cuts in skin	50	2	8.3	10.5	33.0
		Via undamaged skin	50	0	0.0	-	-
(ii)	24	Feeding	50	7	16.7	14.3 (3.9)	45.3 (14.9)
		Injection	50	28	100.0	6.5 (1.5)	16.6 (7.7)
		Via cuts in skin	50	8	28.6	10.4 (2.2)	32.0 (10.5)
		Via undamaged skin	50	1	2.2	12.0	31.0
(iii)	45	Feeding	40	1	3.0	28.0	43.0
		Injection	40	22	100.0	7.1 (1.8)	26.1 (11.9)
		Via cuts in skin	40	3	8.1	12.0 (3.3)	22.7 (2.6)
		Via undamaged skin	28	0	0.0	-	-
(iv)	47	Feeding	50	4	8.2	9.0 (1.6)	42.0 (13.7)
		Injection	50	38	95.0	5.7 (1.1)	24.4 (8.4)
		Via cuts in skin	50	5	11.4	11.6 (5.5)	33.4 (19.2)
		Via undamaged skin	50	2	4.0	9.0	22.0

*1 The percentage of larvae becoming infected was corrected by discounting the deaths of those larvae which died without developing iridescence before the day of the mean incubation period for that treatment.

*2 Standard deviations are given in brackets.

*3 Seventeen-day old larvae were too small for injection.

Table 19. Levels of significance for differences in incubation period between treatments.

	Via cuts in the skin	Feeding
Injection	0.001 (ii) 0.001 (iii) 0.001 (iv)	0.001 (ii) 0.01 (iv)
Via cuts in the skin		0.01 (ii) Not significant (iv)

The test numbers are given in brackets. Treatments where very few larvae became infected are not included.

Table 20. Levels of significance for differences in survival time between treatments.

	Via cuts in the skin	Feeding
Injection	0.001 (ii) not significant (iii) not significant (iv)	0.001 (ii) 0.01 (iv)
Via cuts in the skin		0.02 (ii) Not significant (iv)

The test numbers are given in brackets. Treatments where very few larvae became infected are not included.

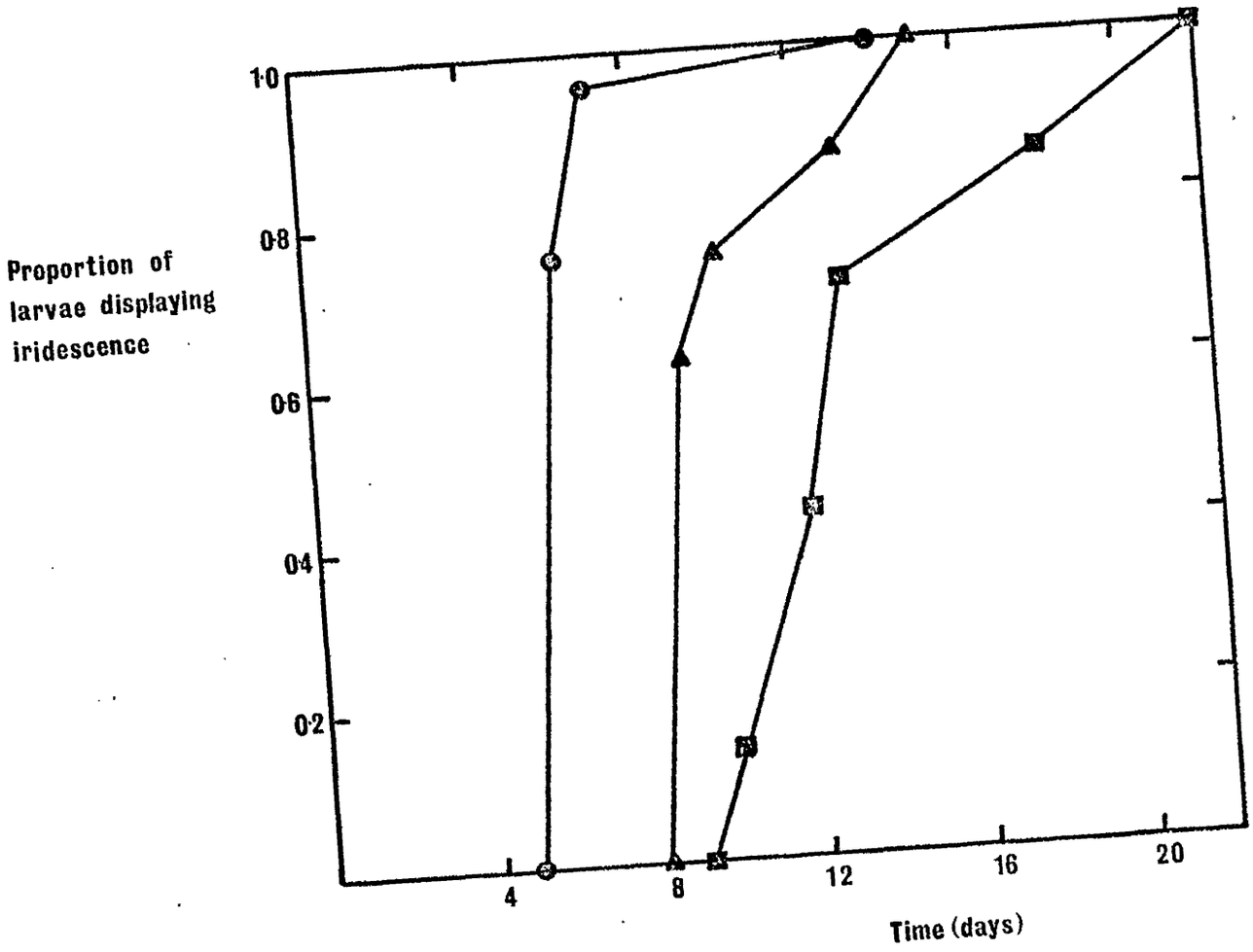


Fig.17. Rates of appearance of iridescence in larvae inoculated with TIV by different routes. Data from test (ii).

injection

via skin wounds

feeding

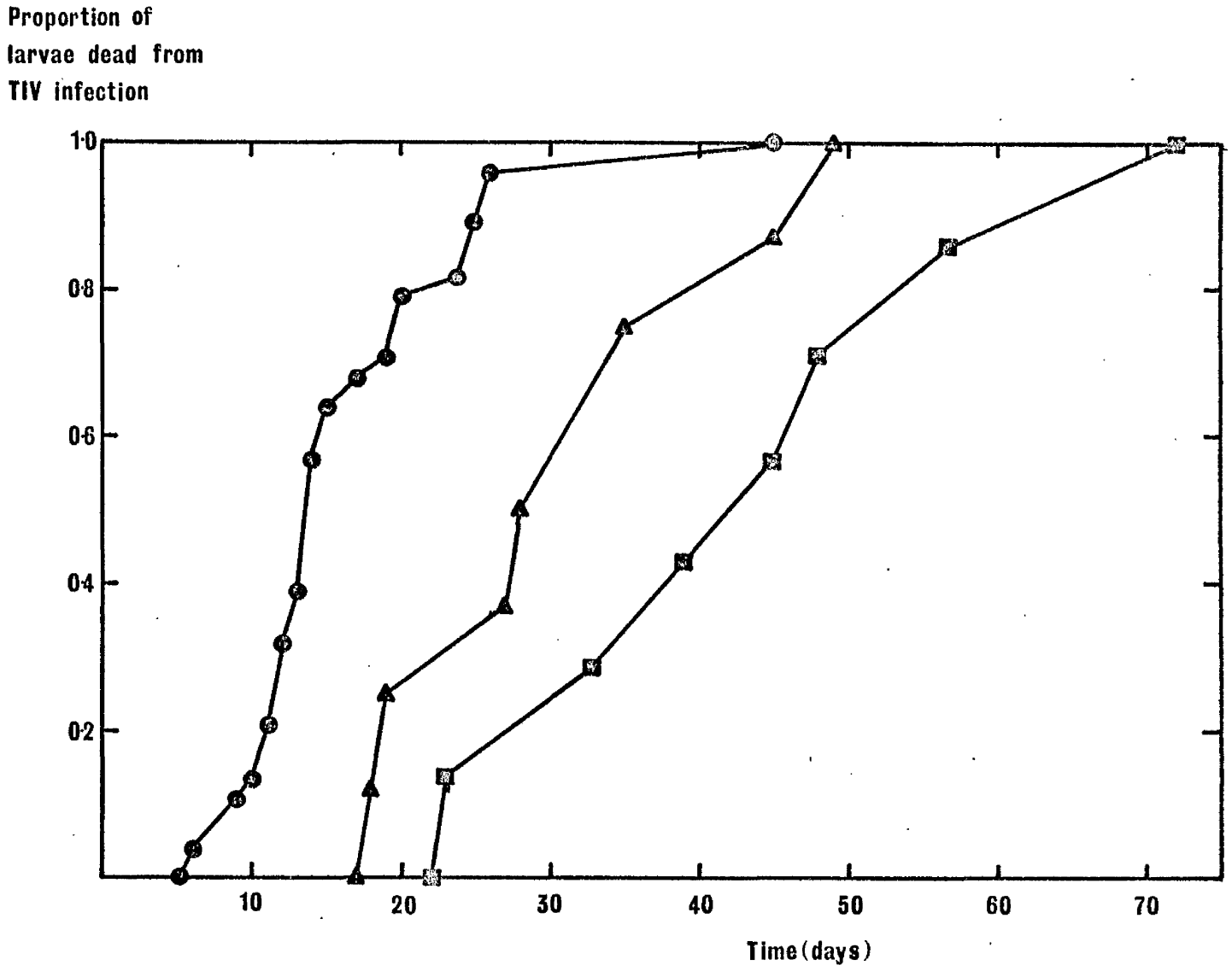


Fig.18. Death rates of larvae inoculated with TIV by different routes.

Data from test (ii)

injection

via skin wounds

feeding

Discussion

These results confirm that TIV has a low infectivity when taken orally by leatherjackets, but a high infectivity when it enters the haemocoel directly. Similar findings have been made in several other insect/virus systems. Stairs (1965a) found that the same dosage of NPV resulted in lower mortality of Galleria mellonella larvae when given orally than when injected. Honey bees are much less susceptible to both the chronic and acute bee paralysis viruses by feeding than by injection (Bailey, 1965a; Gibbs, 1968), and the silkworm shows a similar pattern with its NPV (Aizawa, 1962) and CPV (Miyajima and Kawase, 1967). Martignoni (1957), however, found that the granulosis virus of Eucosma griseana (Hübner) did not cause infection when injected into the haemocoel of the larva, but did when administered per os; it may be that breakdown of the inclusion bodies in the gut to liberate free virions was essential in this case. Fukaya and Nasu (1966) found that larvae of Chilo suppressalis were readily infected with CIV, both by feeding and by injection.

The reasons why many insect viruses have a low infectivity when administered orally are uncertain. Chamberlain and Sudia (1961), in discussing the infection of mosquito vectors with vertebrate viruses, mention several hypotheses, including: virus inactivation by digestive fluids; impermeability of the peritrophic membrane; and a limited number of specific virus receptor sites on the gut cells. Schildmacher (1950) showed that colloidal gold particles, 2 - 4 nm in diameter, diffuse through the peritrophic membrane of mosquito larvae, but particles measuring

20 nm do not. It is unlikely that the TIV particle, the diameter of which is at least 6 times greater than this, is able to cross the peritrophic membrane of the leatherjacket by the normal processes of diffusion.

With some insect viruses there is evidence that, after entering the insect *per os*, the gut cells are infected before the virus enters the haemocoel and infects other organs, e.g. the granulosis virus of *Trichoplusia ni* (Summers, 1969) and the NPV of the small tortoiseshell butterfly, *Aglais urticae* (Linnaeus) (Harrap and Robertson, 1968). Possibly a similar mechanism operates with TIV; it may be that the insect excretes most of the virus before it infects the gut cells. There was no evidence that the larval gut fluids lower the infectivity of TIV (Experiment 15).

The majority of larvae inoculated with TIV via cuts in the skin did not become infected, in spite of a large dose being present in the inoculum. The probability of larvae coming into contact with large doses of TIV immediately after fighting and sustaining damage to their skins is fairly remote, so this mode of infection is unlikely to be of much significance in nature. When TIV-infected and healthy larvae were confined together (Experiment 8) there was no evidence of transmission of the virus in this manner.

Very few larvae became infected when virus was dropped onto undamaged skins. Normally there is a flow of water in through the cuticle and out through the anus, probably involving the malphigian tubules (A. Neats, personal communication), so if the pores in the skin are large enough, virus could possibly have entered by this route, but it is probable that these few larvae became infected *per os*.

Although leatherjackets have a high resistance to TIV when it enters the insect *per os*, this may be the natural route of infection, and *Tipula* may have developed this resistance during its evolution. The reason why the insect has not developed immunity to direct infection via the haemocoel suggests that infection via this route rarely occurs in nature. Epizootics of TIV in tipulid larvae are rarely observed, and this may reflect the fact that the transmission of the virus is not readily accomplished. If *per os* is the normal route of infection, then the larva must ingest a massive dose of TIV in order to become infected.

There was much variation in both incubation period and survival time, especially the latter, but both tended to be shorter with more efficient inoculation routes. Presumably with less efficient inoculation routes fewer virions gain access to susceptible cells, so that more replicative cycles of the virus must occur before the effects of the disease become apparent. The pattern was similar to that in Experiment 2, where larger doses of TIV tended to result in shorter incubation periods and shorter times to death.

The LT50 of TIV has been given as 14 - 28 days (Rivers and Glitz, 1967, personal communication to Ignoffo, 1968); these values were presumably obtained by injecting virus. The mean times to death from TIV administered by injection in the present experiment were 16.6, 26.1 and 24.4 days, but the corresponding mean times to death from TIV administered by feeding were 45.3, 43.0 and 42.0 days.

Experiment 14. A comparison of techniques for inoculating leatherjackets with TIV per os.

Introduction

When investigating the effects of temperature on the infection (Section D) it was planned to study insects infected with TIV both by injection and per os. It was shown (Experiment 13) that only a small proportion of leatherjackets become infected when they drink from a concentrated TIV suspension, so the present experiment was performed to determine whether a more efficient technique for inoculating TIV per os could be found.

Methods

The following inoculation techniques were tested:

a) Each larva was placed in a dry petri dish, and left for 4 h to desiccate, then 0.005 ml TIV suspension was placed in each dish. Most of the larvae drank from the drop. They were left for a further 2 h, then each was provided with a filter paper, grass and SVS. This was identical to treatment a) in Experiment 13.

b) In the dish of each larva was placed a small amount of powdered grass which had been moistened, and to which had been added 0.005 ml TIV suspension. Each larva was given fresh food only when this first portion had been eaten.

c) The needle of a hypodermic syringe was forced between the mandibles, and into the mouth of each larva, after it had been anaesthetised, and 0.005 ml TIV suspension was delivered directly into the gut.

In one test 100 fourth-instar larvae were used for each treatment, and each insect received a dose of 4.8×10^5 LD50 units (by injection) TIV.

Larvae and pupae which died without developing iridescence were tested for infection with TIV by centrifugation of extracts (see Materials and Methods).

Results and Discussion

None of the 3 techniques were very successful in infecting leatherjackets with TIV per os (Table 21). Introducing virus directly into the gut using a hypodermic syringe was 3 to 4 times more efficient than allowing the larva to drink from a TIV suspension (the technique employed in Experiment 13). Mixing virus with the food resulted in a very small proportion of insects becoming infected.

The efficiency of each technique may reflect the time period over which the inoculum passed through the gut. With the most efficient technique (direct introduction into the gut) all the inoculum was present in the gut at the same time, whereas with the least efficient technique (mixing virus with the food) some virus had possibly already been excreted by the larva before all of the inoculum had been ingested. The situation with drinking from a drop of TIV suspension is intermediate between these two, although more similar to the latter, this being reflected in the number of insects which became infected.

It was therefore decided to use treatment c) for inoculating larvae per os in Section F, although it must be borne in mind that the insertion of a syringe needle into the larval gut might damage the tissues, and thus aid the penetration of the virus into the haemocoel.

The results were similar to those of Experiment 13, in that the most efficient inoculation route tended to result in shorter incubation periods and shorter survival times (Table 21).

Table 21. Results of inoculating leatherjackets with TIV per os using different techniques.

Test Number	Treatment *1	No. of larvae inoculated	No. of insects becoming infected with TIV	Corrected percentage infection*2	Mean incubation period (days)*3	Mean survival time (days)*3
(i)	a	100	4	4.0	11.5 (2.6)	34.5 (14.6)
	b	100	2	2.0	7.0	9.5
	c	100	13	14.4	9.4 (2.0)	32.7 (7.6)
(ii)	a	50	4	8.2	9.0 (1.6)	42.0 (13.7)
	c	50	13	26.0	7.5 (1.5)	37.3 (7.5)

*1 See text for explanation of treatments.

*2 The percentage of larvae becoming infected was corrected by discounting those larvae which died and were found to be TIV-negative up to 2 days before TIV symptoms first began to appear in that treatment.

*3 Standard deviations are given in brackets. Cases where infection was not observed until the pupal or adult stages are not included.

In some insects, TIV symptoms were not observed until the pupal or adult stages (see Experiments 18 and 19).

Experiment 15. To determine whether the larval gut fluids affect the infectivity of TIV.

Introduction

It has been demonstrated (Experiments 13 and 14) that TIV has a low infectivity when it enters a T. oleracea larva per os. This may possibly be due, wholly or partly, to the presence of some antiviral substance(s) in the larval gut fluids. There are very few reports on the examination of insect gut fluids for the presence of antiviral factors. Aizawa (1962) found that silkworm NPV is inactivated in vitro in the presence of the gut fluids of silkworm larvae, and Hayashiya, Nishida and Matsubara (1969) isolated a protein with antiviral activity from the gut juice of silkworms. Chamberlain and Sudia (1961), however, found that the digestive enzymes of mosquitoes had little effect on the virus titre of eastern encephalitis virus.

The pH of the larval gut fluids was first determined, then they were incubated with a TIV preparation, and tests were performed to determine whether the infectivity of the virus was reduced.

a) Measurement of the pH of the larval gut fluids.

The method was based upon that of Day and Powning (1949) who measured the pH in the gut of the cockroach, Blattella germanica (Linnaeus), by incorporating pH indicators with a diet of starch or gelatin. It was found that T. oleracea larvae thrived on a diet of boiled, mashed potato, so various pH indicators were incorporated with this diet, and the colours of the indicators were observed through the skin. Day and Powning (1949) found it necessary to puncture the gut of Blatella germanica in order to observe the colour of the contents.

The pH range over which the indicators changed colour was determined (Table 22) using a pH meter, and varying the pH by either adding dilute HCl to dilute NaOH, or *vice versa*. Each indicator was fed to 5 fourth-instar larvae. All 5 larvae did not always show the same reaction with the same indicator, and sometimes there were differences in individual larvae with time. Table 23 shows a typical set of results, 12 days after the larvae began to feed on the potato/indicator mixtures. In some cases the anterior tip of the gut showed a different colour to the remainder of the anterior portion. Readings taken on other days sometimes showed slight variations from the results quoted in Table 23, but the basic pattern was similar, i.e. the anterior tip of the gut was weakly alkaline, followed by a portion which was strongly alkaline, and the posterior section was acid.

In order that the colours of the different portions of the gut could be seen more clearly, some of the leatherjackets were dissected after they had been feeding on the potato/indicator mixtures for 18 days. The morphology of the gut of *T. oleracea* was observed to be very similar to that of *T. paludosa*, as described by Vickerman (1960) (Fig. 19). The results observed during the dissections are recorded in Table 24.

The reaction of the larval gut, then, appears to vary from pH >8.5 in the cardia-ventriculus region to pH 5.7-5.9 in the rectal ampulla. Day and Powning (1949), however, found an increase in pH along the gut of *Blatella germanica*; a carbohydrate diet reduced the pH of the crop contents, but did not change those of the midgut or hindgut. Heimpel

Table 22. Indicators used in the determination of the pH of the larval gut.

Indicator	Concentration (%)	Colour Change	pH range of colour change
methyl red	0.01	red - yellow	5.0 - 5.6
chloro-phenol red	0.04	yellow - violet/red	5.4 - 5.9
bromo-cresol purple	0.04	yellow - violet	5.6 - 6.0
bromo-thymol blue	0.04	yellow - blue	5.7 - 7.4
litmus	0.4	red - blue	6.3 - 6.9
diphenol purple	0.025	yellow - purple	7.0 - 7.6
phenol red	0.02	yellow - purple/pink	7.1 - 7.6
meta-cresol purple	0.04	yellow - violet	7.8 - 8.2
phenolphthalein	0.1	colourless - red	8.1 - 8.3
thymol blue	0.04	yellow - blue	8.2 - 8.4
ortho-cresol phthalein	0.04	colourless - violet/red	8.5 - 8.7

Table 23. pH readings in different sections of larval guts, as observed through the skin.

Indicator	Section of the gut		
	Anterior tip	Anterior	Posterior
methyl red	>5.0	>5.0	>5.0
chloro-phenol red	>5.4	>5.4	<5.9
bromo-cresol purple	>5.6	>5.6	<6.0
bromo-thymol blue	>5.7	>5.7	>5.7
litmus	>6.3	>6.3	? *1
phenol red	>7.1	>7.1	>7.1*2
diphenol purple	<7.6	>7.0	<7.6
meta-cresol purple	<8.2	>7.8	<8.2
phenolphthalein	<8.3	>8.1	<8.3
thymol blue	<8.4	>8.2	<8.4
ortho-cresol phthalein	<8.7	>8.5	<8.7
Overall result	7.1 - 7.6	>8.5	5.7 - 5.9

In cases where not all 5 larvae on 1 indicator showed the same result, the result of the majority is given.

*1 The posterior sections of the larval guts containing litmus were yellow.

*2 Anomalous result.

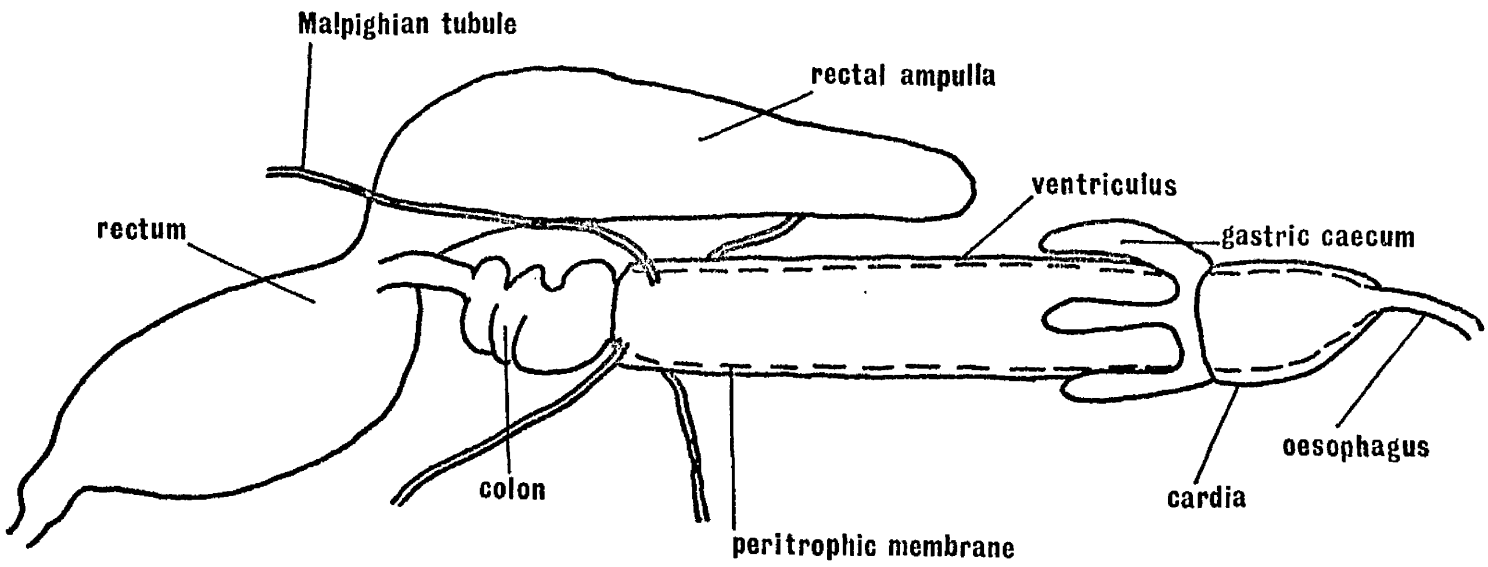


Fig. 19. Morphology of the gut of T.paludosa and T.oleracea (from Vickerman,1960).

Table 24. pH readings in different sections of larval guts, as observed on dissection.

Indicator	Section of the gut							
		Cardia		Ventriculus				
	Oesophagus	Anterior	Posterior	Anterior	Posterior	Colon	Rectum	Rectal ampulla
chloro-phenol red	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4	5.4-5.9
bromo-thymol blue	5.7-7.4	5.7-7.4	5.7-7.4*2	5.7-7.4*2	5.7-7.4	5.7-7.4	5.7-7.4	5.7-7.4
litmus	? *1	? *1	>6.3	>6.3	>6.3	>6.3	>6.3	? *1
phenol red	>7.1	<7.6	>7.1	>7.1	>7.1	>7.1	>7.1	7.1-7.6*4
diphenol purple	<7.6	<7.6	>7.0	>7.0	<7.6*3	<7.6	<7.6*3	<7.6
meta-cresol purple	<8.2	<8.2	>7.8	>7.8	>7.8*3	<8.2	>7.8*3	<8.2
phenolphthalein	<8.3	<8.3	>8.1	>8.1	<8.3	<8.3	<8.3	<8.3
thymol blue	<8.4	<8.4	>8.2	>8.2	<8.4	<8.4	<8.4	<8.4
ortho-cresol phthalein	<8.7	<8.7	>8.5	>8.5	<8.7	<8.7	<8.7	<8.7
Overall result	7.1-7.6	5.7-7.6	>8.5	>8.5	7.6-7.8	7.1-7.6	7.6-7.8	5.7-5.9

*1 These sections of the gut of the larva fed litmus were yellow.

*2 Anomalous result.

*3 <7.6, >7.8 interpreted as 7.6-7.8.

*4 This result was assumed to be anomalous in view of the results obtained previously (Table 23), although solely on the basis of the above results that for chlorophenol red could be assumed to be anomalous, giving a value for the rectal ampulla of pH 7.1-7.4.

(1955) studied the gut pH of 11 species of Hymenoptera and 2 Lepidoptera, and found that it changed during development and after starvation. It is not known whether the pH values determined here for T. oleracea larvae feeding on potato also hold true for larvae feeding on powdered grass or a natural diet.

b) Incubation of TIV with larval gut fluids

Methods

A suspension of TIV in distilled water was passed successively through 2.0, 0.8, 0.45 and 0.2 µm membrane filters, which had been pretreated with serum, to remove bacteria and fungi from the suspension (Experiment 6). The filtrate was tested for the presence of bacteria and fungi by inoculating drops on to plates of nutrient agar and malt extract agar, which were then incubated at 20°. There was no growth of bacteria or fungi on the plates after incubation for 4 days.

The guts were dissected out of 120 fourth-instar larvae which had been anaesthetised and decapitated. They were washed in phosphate-buffered saline (pH 7.0), collected over an ice-bath, ground using a pestle and mortar, and diluted with phosphate buffer (pH 7.0) at a rate of 0.1 ml per gut. This preparation was clarified by centrifugation at 11,000 g for 20 min, and then sterilised by passing successively through 2.0, 0.8 and 0.2 µm membrane filters.

The gut juice and TIV suspension were mixed in a 1 : 1 ratio, and incubated at 20°, along with controls consisting of a) gut juice diluted 1 : 1 with sterile distilled water, and b) TIV suspension diluted 1 : 1 with sterile phosphate buffer, pH 7.0. The infectivities of the TIV

plus gut juice and the TIV plus buffer mixtures were assayed by the response time method after 1, 24, 48, 72 and 168 h; larvae were inoculated with 10^{0} and 10^{-2} dilutions, 20 larvae being inoculated with each dilution. Also, at each of these times, 2 larvae were injected with the gut juice plus water mixture.

Results

The results of the infectivity assays (Table 25) suggest that the infectivity of the TIV + buffer mixture remained constant throughout the period of the experiment. The results for the TIV + gut juice mixture are more difficult to interpret, as there is an apparent increase followed by a decrease in infectivity. A t test carried out on the results indicates no significant difference in incubation period between each pair of results, except for those at 168 h, 10^{0} dilution ($p < 0.05$). However, as most larvae injected with the 10^{0} dilution of the TIV + gut juice mixture at 168 h died soon after injection (see below) too much weight is not placed upon this difference in incubation period, especially as there was no significant difference between the incubation periods for the 10^{-2} dilutions at 168 h.

Development of a toxic factor

The numbers of larvae dying in the first few days after injection, before any started to display iridescence, suggested that a toxic factor developed in the TIV + gut juice mixture (Table 26) between 72 and 168 h after the start of the experiment. It was suspected that this toxic factor might be a microbial contaminant as the TIV + gut juice mixture had become cloudy over the 7 days of the experiment, so plates of yeastrel

Table 25. Infectivity assays of TIV + gut juice and TIV + buffer mixtures.

Time (h)	Mixture assayed	TIV titre (LD50 units/ml)
1	TIV + gut juice	4.7×10^5
	TIV + buffer	6.5×10^6
24	TIV + gut juice	1.0×10^6
	TIV + buffer	2.1×10^6
48	TIV + gut juice	1.9×10^7
	TIV + buffer	6.8×10^6
72	TIV + gut juice	7.3×10^5
	TIV + buffer	1.5×10^6
168	TIV + gut juice	4.6×10^5
	TIV + buffer	2.5×10^6

milk agar were streaked from the following preparations: a) the original filtered TIV suspension, b) the filtered gut juice, c) the TIV + gut juice mixture, d) the TIV + buffer mixture and e) the TIV + water mixture.

Table 26. Numbers of larvae injected with TIV + gut juice which died in n - 1 days (where n is the shortest incubation period for that dose - group).

Inoculum	Deaths (out of 20) at assay time (h): 1	24	48	72	168
TIV + gut juice, 10^{0}	0	1	0	3	14
TIV + gut juice, 10^{-2}	0	0	1	2	0
TIV + buffer, 10^{0}	1	0	0	2	1
TIV + buffer, 10^{-2}	5	1	0	4	1
gut juice + water, 10^{0}	0	1	1	1	1

After incubation at 20^{0} for 24 h there was bacterial growth on all the plates which had been streaked with mixtures containing gut juice. Incubation for a further 7 days did not result in any growth on the plates which had been streaked from the other 2 preparations. It was concluded that there was a bacterial contaminant in the gut juice, and that this may have been the reason for the high death rate from 'unknown causes' in larvae injected with the TIV + gut juice mixture after 7 days. Although the TIV preparation had been tested for the presence of bacteria and fungi before the commencement of the experiment, it was not practicable to do so with the gut juice, as the hypothetical antiviral factor(s) might have been labile, and might have become inactivated while waiting for results.

Discussion

This experiment did not detect any effect of the larval gut fluids upon the infectivity of TIV. It is possible that antiviral substances are present in the larval gut, but that they are labile, and became inactive soon after extraction. Alternatively, the pH and temperature of the present test may not have been within the range of the activity of the hypothetical substance(s). It would be interesting to repeat the experiment at different temperatures and at different pHs, as the pH of the larval gut fluids has been shown to extend over a wide range. The development of the 'toxic factor' in the present experiment demonstrates the importance of freeing the virus - gut juice mixture from bacteria and fungi.

Experiment 16. Tests to determine whether the embryo is susceptible to infection with TIV.

Introduction

Laughlin (1958b) observed that the new-laid egg of T. oleracea is shrunken, but by the third day is considerably bigger due to absorption of water. This was confirmed for both T. oleracea and T. paludosa by Meats (1967), who showed that fertile eggs increase their weight by about 50% due to the uptake of water. The possibility that virus might enter the egg with this water and set up an infection was investigated. It was also considered possible that TIV might enter the egg via the micropyle, a specialised region in the membranes of most insect eggs to allow the entry of sperm (Counce, 1961).

Preliminary experiment

1 ml of a TIV suspension, containing approximately 1.0×10^8 LD50 units/ml was spread evenly over the surface of a 9 cm diameter plate of 1% ionagar, and a fertilised female crane-fly was allowed to lay her eggs in the agar. When the larvae had hatched they were removed from the agar, transferred to a pot of sand, and observed regularly. Many larvae died from TIV infection in all 4 instars, while a few did not become infected, but pupated and gave rise to adults. TIV-infected first-instar larvae are shown in Fig. 20. The question arose as to whether the virus had entered the egg and infected the embryo, or whether the larvae had become infected after hatching, from the TIV in the agar.

Main experiment

In order to investigate these 2 possibilities, more female crane-flies

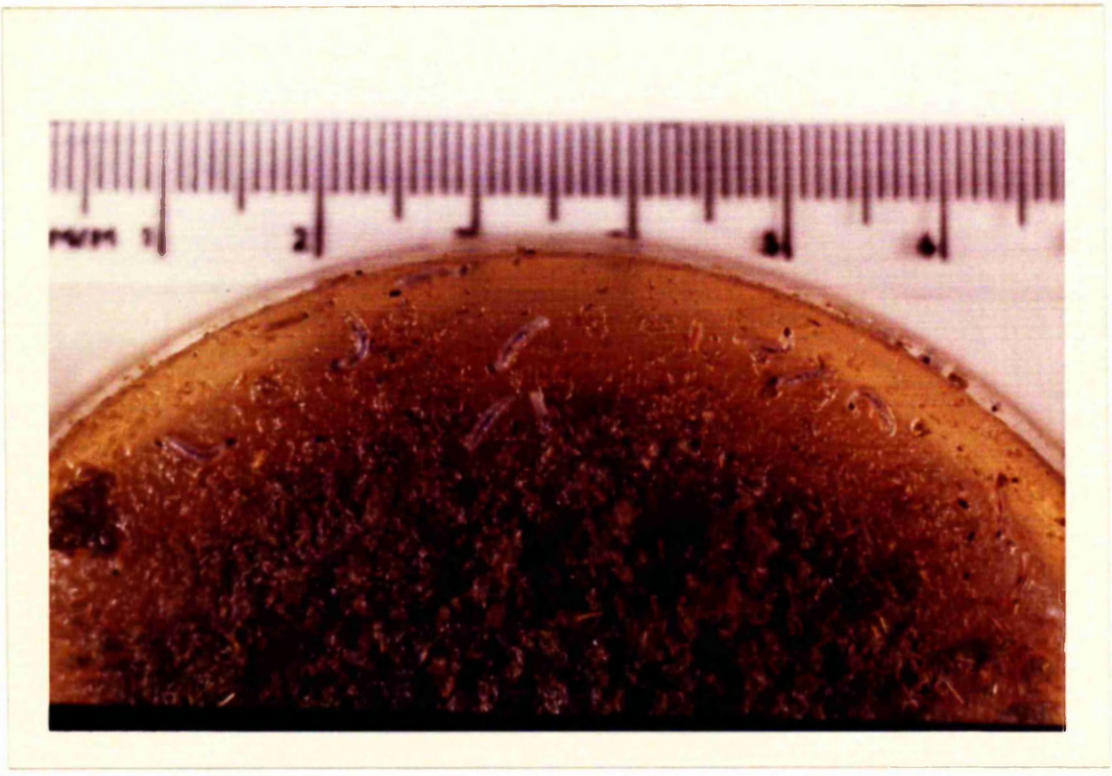


Fig. 20. First-instar T. oleracea larvae infected with TIV

were allowed to lay their eggs in agar containing TIV; 5 plates of fertilised eggs were obtained. On each day before the eggs hatched a sample of 10 eggs was removed from each plate; their surfaces were sterilised by immersion in 10% w/v formalin for 60 min, then they were washed 3 times in distilled water, and transferred to clean agar plates to hatch. Batches of larvae which hatched into agar containing TIV became infected, whereas none of those which hatched from eggs which had been removed from the TIV became infected. It would appear, then, that TIV did not enter the egg and infect the embryo, but that the newly-hatched larvae became infected, probably by ingesting agar containing the virus.

An experiment was also performed to determine how the later larval instars had become infected. Did they contract an infection from the agar in the first instar which did not become overt until a later instar, or did they contract it from other larvae? Eggs were allowed to hatch in a plate of agar containing TIV. The newly-hatched larvae were allowed to crawl in the agar for approximately 24 h, then 50 were picked off and placed in individual petri dishes. Thirty-two of these died in the first instar without developing TIV symptoms; most of them died because they crawled from the moist filter paper and became desiccated. Thirteen larvae (68.4%, correcting for the early mortality due to desiccation) died with TIV symptoms in the first instar, 3 died in the second and third instars without developing TIV symptoms, and 2 pupated and gave rise to adults.

The mean incubation period for those first-instar larvae which became infected with TIV was 13.2 days, this being generally longer than the

incubation periods in fourth-instar larvae inoculated with TIV per os (Experiments 13 and 14), although it was often difficult to observe the iridescence in the first instar due to the small size of the larvae. The mean time to death from TIV was 18.9 days, and the longest time was 31 days. When observing the development of healthy larvae (Experiment 7) there were none remaining in the first instar after day 22; 3 of the 13 larvae (23.1%) which became infected in the present experiment did not die until after day 22.

Discussion

This experiment has demonstrated that the embryo is not susceptible to infection with TIV from the environment; the possibility of the embryo receiving a TIV infection from the parents was studied in Experiment 20. It has been shown, however, that first-instar larvae are susceptible to infection. It is probable that the larvae in this experiment became infected by ingesting the agar containing TIV. As the larvae are so small, they could have ingested only a small dose of TIV, but 68.4% became infected, compared with much smaller percentages in fourth-instar larvae inoculated with large doses of TIV per os (Experiments 13 and 14). This suggests that the larvae are more susceptible to infection with TIV per os in the first instar than in the fourth-instar. Silkworm larvae have been shown to be highly susceptible to infection with NPV when newly-hatched, with susceptibility decreasing markedly as they increase in age (Kobayashi, Yamaguchi and Yokoyama, 1969).

This experiment has also shown that later larval instars may become infected from earlier instars, and that infection of first-instar larvae with TIV may prevent them from passing into the second instar.

Experiment 17. Tests to determine whether leatherjackets can become infected with TIV via the spiracles.

Introduction

Few workers appear to have considered the spiracles as a route of infection for insect pathogens, although many pathogens of vertebrates are known to enter their host via the respiratory route. Bailey (1965b) demonstrated that bacteria and viruses may invade the honey bee via the spiracles. Presumably the organisms are either suspended in aerosol droplets, or are dry and adhering to dust particles.

Attempts were made to inoculate fourth-instar larvae with TIV via the spiracles by various techniques. Introducing virus into the spriacles with a hypodermic syringe, and dipping the posteriors of the larvae into virus suspension were of little value as techniques because the larvae were so active. Anaesthetising them with ether resulted in them closing up their spiracles.

Experiment 17a.

An attempt was made to block the mouths of the larvae by dipping their anterior ends in molten paraffin wax (melting point 46°) at 50°. It was found that if the wax coating extended more than half-way along the length of a larva it was very difficult for it to wriggle free, so larvae were coated in wax almost right up to the spiracles. Larvae which had been coated with paraffin wax in this way were subjected to the following three treatments, 100 larvae being used for each treatment:
1) About 1 ml TIV suspension in ISA (approximately 1.0×10^8 LD50 units/ml) was sprayed from a spray gun (Shandon Scientific Co. Ltd.), to form an

aerosol just above the surface of the larvae which were laid out over an area of about 0.1 m^2. The larvae were left for 10 min to 'breathe in' the virus.

2) 1 ml TIV suspension in ISA was dried, and made into a dust. Each larva had a small amount of this dust brushed onto its spiracles.

3) The spiracles of each larva were held beneath the surface of a TIV suspension in ISA for 3 min.

Each treatment was also carried out with 10 control larvae, but with ISA in place of the TIV suspension. After the treatment, each larva was washed 3 times in distilled water, and then released from the paraffin wax.

None of the larvae developed iridescence, but latex tests were carried out on all dead larvae, pupae and adults, and a few positives were found (Table 27). Large numbers of larvae died as a result of the treatment with paraffin wax.

Table 27. Results of attempts to infect leatherjackets with TIV via the spiracles, with their mouths blocked with paraffin wax.

Treatment	No. becoming infected/ no. surviving after sixth day	Percentage infection
1) (spray)	2/57	3.5
2) (dust)	0/67	0
3) (dip)	4/38	10.5
1) (spray) control	0/8	0
2) (dust) control	0/7	0
3) (dip) control	0/7	0

The results suggest that a few of the larvae which were sprayed with, and dipped in, virus may have become infected via the spiracles. It was not certain, however, that the paraffin wax had effectively blocked the mouth, and as the highest rate of infection came from larvae dipped in virus, it was considered possible that the liquid may have seeped between the paraffin wax and the skin of the insect, and entered per os. There is also a slight possibility that the virus passed through the skin in some way. It is not known to what extent the hot wax treatment might have enhanced or interfered with infection.

Experiment 17b

The second approach was to try to block the guts of one group of larvae, and the spiracles of another group by ligaturing the insects, which were then exposed to TIV to determine whether there was a difference in infection rate between the 2 groups.

Three hundred larvae were ligatured with alginate thread, half of them anteriorly, just behind the head, and the other half posteriorly, just before the spiracles. The larvae were anaesthetised with ether just before ligaturing, and then left for about 30 min for the effects of the anaesthetic to wear off. They were then exposed to TIV by treatments 1) - 3) of Experiment 17a, 50 anteriorly-ligatured and 50 posteriorly-ligatured larvae being used for each treatment.

After being treated, each larva was washed twice with water to remove TIV from the skin, and then immersed in 1% sodium hexametaphosphate solution to dissolve the alginate thread. This technique for ligaturing the larvae was found to be far superior to the use of cotton thread, which

had to be loosened from the larvae with the aid of dissecting needles, the insects often becoming damaged in the process.

To test whether the ligature affected the development of the disease in any way, 60 larvae were each injected with 0.005 ml TIV suspension; 20 had been ligatured anteriorly, 20 posteriorly, and 20 were not ligatured. The ligatures were removed immediately after the larvae had been injected.

Some larvae developed iridescence, and in some TIV was detected after death by the latex test, which was carried out on all insects which died without TIV symptoms; TIV was detected in two adults (see Experiment 19). The results are summarised in Table 28, together with the results of the χ^2 test which was carried out on each pair of data. These show that in each treatment there was no significant difference in the number of insects becoming infected, between those ligatured anteriorly and those ligatured posteriorly. Therefore, it has not been shown whether the mouth or the spiracles was the route of infection.

The incubation periods for the injected larvae are given in Table 29. The t test indicates that there is no significant difference in incubation period between the treatments, so there is no evidence that the ligatures had interfered with the course of infection.

Five of the larvae which had been ligatured anteriorly (1, 2, and 2 larvae from treatments 1), 2) and 3) respectively) did not pupate normally. The cuticle became dark brown and hard like that of a pupa, but the larval skin was not lost. Presumably the ligature had interfered with the normal moulting processes, perhaps by interfering with the transport of hormones within the larva.

Table 28. Results of exposing leatherjackets to TIV with some ligatured anteriorly and some ligatured posteriorly.

Treatment	No. becoming infected/Total*	Percentage infection
1) (spray) anterior ligature	0/36	0.0
posterior ligature	2/33	6.1
		$\chi^2 = 0.609$
2) (dust) anterior ligature	1/45	2.2
posterior ligature	2/41	4.9
		$\chi^2 = 0.007$
3) (dip) anterior ligature	5/33	15.2
posterior ligature	5/42	11.9
		$\chi^2 = 0.005$

* Those insects which died and were found to be TIV-negative up to 2 days before TIV symptoms first began to appear in that treatment-group are discounted.

Table 29. Incubation periods of ligatured and unligatured larvae injected with TIV.

Treatment	No. of larvae	Incubation periods (days)	Mean incubation period (days)
anterior ligature	19	1x5; 8x6; 7x7; 2x8; 1x9	6.68
posterior ligature	17	1x5; 5x6; 5x7; 5x8; 1x10	7.06
no ligature	18	2x5; 4x6; 5x7; 3x8; 3x10; 1x14	7.61

Discussion

Although a few insects became infected with TIV in these experiments, it was not demonstrated that TIV entered the larvae via the spiracles, and it is quite possible that infection occurred *per os*. The possibility that the treatments damaged the skin, allowing direct entry of the virus into the haemocoel must also be considered. None of the treatments resulted in a very high infection rate, and it can be concluded that TIV infects leatherjackets via the spiracles rarely, if ever.

Experiment 18. Inoculation of pupae with TIV.

Introduction

Although insect viruses are usually found infecting their host larvae, there are a number of reports of infections in the pupa. NPVs have been found to infect the pupae of the following insects: Bombyx mori (Aizawa, 1953), Hemerocampa pseudotsugata (McDunnough), Orgyia antiqua (Linnaeus), Lambdina fiscellaria, Peridroma margaritosa (Haworth) (Sager, 1960), Galleria mellonella (Stairs, 1965b), the African armyworm, Spodoptera exempta (Walker) (Brown and Swaine, 1965), Ectropis crepuscularia (Schiff) (Morris, 1967) and Trichoplusia ni (Vail and Hall, 1969a). The pupa of T. ni has also been found to be susceptible to a CPV (Vail, Hall and Gough, 1969).

Among the iridescent viruses, Day (1965) found that final-instar G. mellonella larvae inoculated with SIV pupated, and died from SIV as pupae. Chapman et al (1966) observed that most mosquito larvae infected with MIV died before pupation, but a few pupated and died as pupae. Smith et al (1961) found that many larvae of Porthetria dispar and Pieris brassicae inoculated with TIV in a middle instar, did not die from TIV infection until after pupation. In P. dispar this caused the legs and wing buds to atrophy and exhibit iridescence; electron micrographs of sections of infected pupae revealed large amounts of virus in the wing buds, legs and head. The effect of TIV on prepupae and pupae of P. brassicae was less marked. Some TIV-infected silkworm larvae are able to spin thin-layered cocoons, but they do not undergo the pupal moult (Hukuhara, 1964).

Silkworm pupae, however, can be infected with TIV, and iridescence can be observed in many organs (Hukuhara and Hashimoto, 1966b).

Ahmed (1968) found a TIV-infected Tipula pupa in the field; it had deformed wing buds, and was displaying iridescence. He also successfully infected T. paludosa pupae with TIV in the laboratory. During the present investigations, several larvae which had been inoculated with TIV pupated. Some of the pupae resembled that described by Ahmed (1968), having deformed wing buds and displaying iridescence (Fig. 21); some appeared to be normal pupae, but iridescence could be observed internally upon dissection; in some the pupa was apparently normal, both externally and internally, but a TIV infection was detected by the latex test. In no case did a pupa displaying iridescence give rise to an adult, but several apparently healthy pupae, derived from larvae which had been exposed to TIV, gave rise to TIV-infected adults (see Experiment 19).

Methods

The following techniques used by Ahmed (1968) to inoculate T. paludosa pupae were used for T. oleracea pupae:

a) The pupa was submerged in a TIV suspension for 3 min.

b) The pupa was pricked with a sterile fine pin, just behind the wing buds on the ventral side, then the wound was brushed with TIV suspension.

c) The pupa was injected with 0.005 ml TIV suspension just below the integument in the ventral thoracic region.

Control pupae received similar treatments, but ISA was used in place of the TIV suspension. Pupae were treated as they became available, over a period of about 9 months. The TIV suspension (approximately 1.0×10^8 LD50 units/ml) was stored at -16° over this period. Each treatment was

TIV-infected not infected TIV-infected, recently pupated

Fig. 21. TIV-infected and healthy T. oleracea pupae

Scale : mm.

carried out with 100 pupae, and each control group also consisted of 100 pupae. All pupae and adults which did not develop iridescence were tested for TIV by the latex test.

Results

The numbers of insects becoming infected (Table 30) indicate that the three inoculation techniques can be placed in the following order of efficiency: c>b>a.

Table 30. Numbers of pupae becoming infected after inoculation with TIV by three different methods.

Treatment	Number of insects becoming infected (out of 100)
a (submerged)	8
b (pricked)	52
c (injected)	89

The percentages becoming infected are probably greater than these figures, because they do not take into account deaths which occurred in the first few days due to the trauma of inoculation. It is sometimes difficult to determine whether pupae are dead, as, unlike larvae, they do not always respond to touch.

There were 3 possible fates for each pupa: it could die as a pupa; an adult could partly emerge; an adult could fully emerge. Table 31, which breaks down the results into greater detail, shows that the number of deaths of control pupae in treatments b and c was quite high. Treatments b and c

Table 31. Fates of pupae inoculated with TIV.

Treatment	Pupa died	Adult partly emerged*	Adult fully emerged
a	6/14 (42.9%)	1/7 (14.3%)	1/79 (1.3%)
a control	0/8	0/2	0/90
b	28/61 (45.9%)	21/33 (63.6%)	3/6 (50.0%)
b control	0/37	0/36	0/27
c	68/71 (95.8%)	15/20 (75.0%)	6/9 (66.7%)
c control	0/23	0/12	0/65

Each result is given as number of insects in which TIV infection developed/ total number of insects. The percentages of infected insects are given in brackets.

* Partly emerged adults include those ranging from where only the head emerged from the pupal case to those where the whole of the adult body had emerged, except the tips of the legs.

also appeared to affect the ability of many adults to emerge fully from the pupal case. No control pupae developed a TIV infection.

Among those partly-emerged and fully-emerged adults in which TIV was detected were both males and females. One male and one female were displaying iridescence; the other infections were detected by the latex test.

Discussion

It has been demonstrated that T. oleracea pupae are susceptible to infection with TIV. Usually the pupa is killed by the virus, but an adult may emerge; this adult may show signs of infection or it may be apparently healthy, but infected at a low level with TIV.

TIV-infected Tipula pupae occur in nature (Ahmed, 1968), but these probably arise mainly from the pupation of infected larvae. Perhaps occasionally a pupa might receive a wound in an environment with a high level of TIV, and thus become infected. Another possibility is that pupae might become infected with TIV via their respiratory horns.

Experiment 19. TIV infection in adults.

Introduction

The adult stages of a number of insects, as well as the pupae (Experiment 18), have been found to be susceptible to virus infection. A number of instances have been observed where virus-infected larvae or pupae gave rise to infected adults, e.g. NPV infections of Colias eurytheme (Sager, 1960), Neodiprion swainei (Smirnoff, 1961), Galleria mellonella (Stairs, 1965b), and Diprion hercyniae (Neilson and Elgee, 1968); CPV infections of the armyworm, Pseudaletia unipuncta (Haworth) (Tanada and Chang, 1960), Trichoplusia ni (Vail et al, 1969) and four Lepidoptera spp. (Neilson, 1965); in the field crickets Teleogryllus oceanicus and Teleogryllus commodus infected with a nonoccluded virus (Reingamum, O'Loughlin and Hogan, 1970); and in Drosophila infected with sigma virus (Plus, 1954).

Adult insects of several species have been successfully infected by inoculation with viruses, e.g. NPVs in T. ni (Vail and Hall, 1969b) and Peridroma saucia (Hübner) (Martignoni, 1964), sigma virus in Drosophila (Plus, 1954), sacbrood virus in the honey bee (Bailey, 1969), and several arboviruses in their insect hosts (Chamberlain, 1968).

Day (1965) obtained SIV-infected adults of G. mellonella by inoculating them directly with SIV, or by inoculating pupae, which then emerged as infected adults. Mitsuhashi (1967) observed that the green rice leafhopper, Nephotettix cincticeps (Uhler), infected with CIV could moult, and some individuals could become adults. Linley and Nielsen (1968a) observed that a few Aedes taeniorhynchus larvae inoculated with NIV developed symptoms of the disease very late, and were able to pupate and

give rise to adults. TIV infection has been observed in Pieris brassicae adults (Smith, 1963), and TIV-infected crane-flies can emerge from tipulid pupae inoculated with the virus (Ahmed, 1968; Experiment 18). The following is a record of the infected adults encountered during the present investigations, and the results of TIV assays performed on two of them.

Records of TIV-infected T. oleracea adults.

TIV infections were detected in 57 adults of both sexes (Table 32). The bulk of them were derived from pupae which had been inoculated with TIV. Although a number of inoculated larvae gave rise to infected pupae, very few gave rise to infected adults; amongst those which did, most larvae had become infected with TIV per os, and only one had been infected by injection. All pupae which gave rise to infected adults were apparently healthy; no pupa which developed iridescence gave rise to an adult. Amongst those adults displaying iridescence none had fully emerged from the pupal case; iridescence could be seen along the sides of the abdomen, which was swollen. Amongst those adults which were not displaying iridescence, but in which a TIV infection was detected by the latex test, some had not fully emerged from the pupal case, while others had emerged, and were apparently normal, healthy adults.

Assay of TIV in infected adults

The TIV in 2 of the infected adults (see Table 32) was assayed by the response time method. Each adult was ground in distilled water, using a pestle and mortar, and subjected to 1 cycle of low-/high-speed centrifugation. In both cases this resulted in the blue pellet characteristic of TIV. The pellet was suspended in 1 ml ISA, and decimal

Table 32. Records of TIV-infected adults derived from infected larvae and pupae.

Experiment No.	Treatment of larva/pupa	No. of insects	Sex	Method of TIV detection
10	larva confined with faeces of TIV-infected larvae	1	♀	latex test
11	larva had ingested a TIV-infected larva	3	♀	adult displaying iridescence*1 and iridescent pellet after centrifugation.
		1	♀	latex test.
14	larva had been inoculated with TIV per os	1	♀	adult displaying iridescence*2 and iridescent pellet after centrifugation.
17	larva dipped in TIV	1	♀	latex test
	larva sprayed with TIV	1	♂	latex test
	larva dusted with TIV	1	♂	latex test
18	pupa injected with TIV	1	♂	adult displaying iridescence
	pupa pricked, then brushed with TIV	1	♀	adult displaying iridescence
	pupae inoculated with TIV by injection, pricking + brushing and dipping	5	♂	latex test
		6	♀	latex test
		34	not known *3	latex test
22a	larva injected with TIV	1	not known	latex test

*1 TIV in one of these adults assayed.

*2 TIV in this adult assayed.

*3 The sex of many of these adults was not determined because their abdomens did not fully emerge from the pupal case.

dilutions in ISA were prepared to 10^{-3}. The 10^{-1} and 10^{-3} dilutions were assayed, using 20 larvae for each dilution. The adult from Experiment 14 was found to contain 3.6×10^{7} LD50 units TIV. The larva from which this adult was derived had been inoculated with 4.8×10^{5} LD50 units TIV, so an increase in TIV of approximately 100-fold had occurred. The adult from Experiment 11 was found to contain 2.3×10^{8} LD50 units TIV. The larva from which this adult was derived had fed upon the TIV-infected cadaver of another larva, so the size of the inoculum in this case is not known.

Discussion

The observation of Ahmed (1968) that T. paludosa adults are susceptible to infection with TIV has now been confirmed for T. oleracea. It has also been shown that the fat body of T. paludosa larvae, pupae and adults is susceptible to infection with a rickettsia (Huger, 1959). Ahmed (1968) observed that none of the larvae or pupae displaying iridescence, which he collected from the field, gave rise to adults. During the present investigations it was found that larvae which developed iridescence nearly always died as larvae; occasionally a late fourth-instar larva developed iridescence and was able to pupate, although the resulting pupa was invariably deformed and iridescent, and soon died. An inoculated fourth-instar larva might not develop iridescence until after it pupated, in which case again it died as a pupa. Those inoculated insects which gave rise to infected adults showed no signs of TIV infection either as larvae or pupae.

Ahmed (1968) also inoculated T. paludosa adults with TIV, and claimed that they became infected, but the only evidence to support this was that their bodies became swollen. It would be interesting to inoculate tipulid adults with TIV, and determine whether there was any increase in TIV. Crane flies only live for a few days, so if TIV replication occurs at the same rate as in the larva (Experiment 2) there will not be any vast increase in TIV.

It is unlikely that crane flies in nature become infected with TIV, except by emerging from infected pupae. Crane flies do not feed, although they may occasionally drink water (Barnes, 1937; Coulson, 1962), which is unlikely to contain large amounts of TIV.

Experiment 20. To test whether TIV can be vertically transmitted via the adults.

Introduction

It is important for a parasite to possess an efficient mechanism for vertical transmission, i.e. transmission to the next generation of its host. There is evidence that many insect viruses are passed on to the new generation directly via the adults. If the virus is inside the egg, transmission is referred to as transovarial, but if the virus is only present on the egg surface, transovum transmission is said to occur (Smith, 1967).

Conte (1907) was the first to suggest transmission via the egg, in connection with the silkworm viruses. This has been confirmed for the CPV of the silkworm (Hukuhara, 1962), and also for many other insect/virus systems, e.g. NPVs of Colias eurytheme (Thompson and Steinhaus, 1950), Neodiprion swainei (Smirnoff, 1961), the western tent caterpillar, Malacosoma pluviale (Dyar) (Wellington, 1962), the cotton leafworm, Prodenia litura (Fabricius) (Harpaz and Ben Shaked, 1964), Spodoptera exempta (Swaine, 1966), and Porthetria dispar (Doane, 1969); the CPV of C. eurytheme (Tanada et al, 1964); the granulosis virus of Peridroma margaritosa (Steinhaus, 1947); and sigma virus of Drosophila (Bregliano, 1969).

Many insect vectors of plant viruses support virus replication, and transmission via the egg is common (Maramorosch, 1963), e.g. clover club-leaf virus in the leafhopper, Aqalliopsis novella (Say) (Black, 1949). It appears that arboviruses may be transmitted transovarially in ticks, but not in mosquitoes (Burgdorfer and Varma, 1967).

Among the iridescent viruses Day (1965) bred from SIV-infected Galleria mellonella adults, but none of the progeny developed an SIV infection. Woodard and Chapman (1968) demonstrated that transovarial transmission of MIV occurs in the mosquitoes Aedes taeniorhynchus and Psorophora ferox (Humbold). Anderson (1970) found the iridescent virus of Aedes stimulans in larval ovarian tissue, and suggested that it may be transmitted transovarially. The present experiment investigated the possibility that TIV may be transmitted in this way.

Methods and Results

In a number of experiments, larvae which had been injected with approximately 1 LD50 TIV survived and gave rise to adults, while larvae which had been injected with an identical dose became infected with TIV and died. It was considered possible that some of these adults might have been infected sublethally, and that they might transmit the virus to their progeny. Fifty-eight of these adults, including males and females, were mated with members of the opposite sex which had not been treated with TIV; 34 of these matings resulted in the production of fertile eggs. The progeny were observed regularly for TIV symptoms, but they were not found in any of the insects. Twenty-seven of the adults were tested for the presence of TIV by the latex test after their eggs had been laid. All of the adults tested, including some which produced fertile eggs and some which did not, gave negative results.

A number of pupae inoculated with TIV also gave rise to apparently healthy adults (Experiment 18), 31 of which were mated with untreated insects. Sixteen of these matings resulted in the production of fertile

eggs, and again none of the insects which hatched developed a TIV infection. All of the adults which were mated were tested by the latex test, and two gave positive results. One of these was a female, which died without laying any eggs. The other was a male; the female with which it was mated laid eggs, but they did not hatch.

Discussion

No evidence was obtained for the transmission of TIV from adult tipulids to their progeny via the egg. Rivers (1966b), too, found no evidence for the transmission of TIV in this way, although the NPV of Tipula has been observed in the progeny of crane flies which had survived experiments with NPV, which suggests that this virus may be egg-transmitted (Rivers, 1966a).

In contrast to TIV, the transmission of MIV via the egg seems to be well established, and it has been suggested (Linley and Nielsen, 1968b) that this may be the most important means whereby the virus is transmitted from one generation to the next.

All of the adults from which progeny were obtained in the present experiment gave a negative result in the latex test. It would be interesting to determine whether adults which are latex-positive are able to produce fertile eggs, and if so whether any of the progeny are infected with TIV.

D. EFFECTS OF TEMPERATURE ON TIV INFECTION IN LEATHERJACKETS

Introduction

The effects of both constant and fluctuating temperatures on TIV infections in T. oleracea were studied in an attempt to gain some understanding of how the disease might develop in the field at different times of the year. Information on the effects of temperature on the probability of the insect becoming infected, the replication of the virus, and the time to kill the host is essential if TIV is to be considered as an agent of biological control.

Experiment 21. Effects of constant temperatures on TIV infection in leatherjackets.

Introduction

Virus infections are markedly affected by temperature. Dutky (1959) pointed out that each virus has an optimum temperature, and upper and lower temperature limits. These critical temperatures for the virus may or may not coincide with those of the host, although Stairs (1968) suggested that both virus and host probably have the same optimum temperature. Gravell and Granoff (1970), after studying the effects of temperature on frog polyhedral cytoplasmic deoxyribovirus, suggested that the viruses of poikilotherms replicate with maximum yield over a broader range of temperature than do the viruses of homeotherms, this possibly being due to the fact that the host enzymes function over a wider range of temperature.

The temperature limits and optima of several insect viruses have been studied. Pieris rapae has been found to be unaffected by its

granulosis virus at 36° (Tanada, 1953), Diprion hercyniae by a NPV at 29.4° (Bird, 1955), Macrosteles fascifrons by aster yellows virus at 32° (Maramorosch, 1955b), and Trichoplusia ni and Heliothis zea by NPVs at 39° (Thompson, 1959). The optimum temperature for aster yellows virus replication in M. fascifrons was found to be 25° (Maramorosch, 1955b), and Pimentel and Shapiro (1962) found that the optimum temperature for Galleria mellonella NPV is 27°.

The time taken for a virus to kill its host is dependant upon temperature (Bergold, 1953), and Dutky (1959) has suggested that with many insect viruses the time to death approximates a hyperbolic function of temperature. A decreasing survival time with increasing temperature was found with NPV infections of Colias eurytheme (Thompson and Steinhaus, 1950), Neodiprion sertifer (Krieg, 1955), T. ni (Drake and McEwen, 1959), H. zea (Ignoffo, 1966) and with the fusiform virus of the cockchafer, Melolontha melolontha (Linnaeus) (Hurpin, 1968). Temperature has also been found to affect incubation period (time from inoculation to appearance of symptoms). A decreasing incubation period with increasing temperature was found in silkworm pupae inoculated with NPV (Aizawa, 1953) and in Neodiprion swainei larvae infected with NPV (Smirnoff, 1961).

The susceptibility of the insects to infection does not appear to vary much with temperature within the temperature limits of the virus. Similar mortalities at different temperatures have been recorded for C. eurytheme (Thompson and Steinhaus, 1950), G. mellonella (Pimentel and Shapiro, 1962) and H. zea (Ignoffo, 1966) inoculated with NPVs, and for M. melolontha inoculated with the fusiform virus (Hurpin, 1968).

Some effects of temperature upon the iridescent viruses have been studied. Day and Mercer (1964) inoculated Sericesthis pruinosa and G. mellonella larvae with SIV, and incubated them at 11, 16, 22 and 28°; they reported that virus replication proceeded normally at 16 and 22°, very slightly at 11°, and not at all at 28°. The optimum temperature for SIV varies with different host species. In cell cultures of Antheraea eucalypti it was found to be 20° (Bellett, 1965), while Day and Dudzinski (1966) found it to be 22° in 5 host species, and 25° in 3 others. The optimum temperature of SIV in S. pruinosa, its natural host, was 25°, the same as the optimum for the insect, whereas the optimum in G. mellonella was 22°, although the optimum for this insect is about 35°. Little SIV replication occurred at temperatures above 28°.

Little work has been done on the effects of temperature on CIV infections, but the virus is known to replicate readily in Chilo suppressalis larvae at 25° (Mitsuhashi, 1966) and in G. mellonella larvae at 28° (Cunningham and Hayashi, 1970). Woodard and Chapman (1968) found that an increasing percentage of Aedes taeniorhynchus larvae became infected with MIV with increasing temperature over the range 20 - 30°, which contrasts with the findings for some other insect viruses (see above), and also with the results of Linley and Nielsen (1968a), who found no significant difference in infection rate between larvae incubated at 20° and 30°.

Tanada and Tanabe (1965) studied the effect of temperature on TIV infection in G. mellonella larvae. Inoculated larvae incubated at 23 - 25° died of TIV infection, whereas those incubated at temperatures above 30° survived and produced adults. Oliveira and Ponsen (1966) found that TIV replicated readily in haemocytes of Pieris brassicae incubated at room temperature (10 - 20°).

The present studies were designed to obtain information on the temperature limits and optimum for TIV, and to investigate whether temperature affects the susceptibility of the leatherjacket to infection.

a) Comparison of TIV infection in T. oleracea at 5° and 20°

A preliminary experiment compared the development of the disease at 5° and at 20°. Decimal dilutions of a TIV suspension (approximately 9.6 x 10^7 LD50 units/ml) to 10^{-8} were prepared, and injected into T. oleracea larvae, each dilution being injected into 6 larvae. Three larvae from each dose-group were incubated at 5°, and 3 at 20°.

The numbers of insects becoming infected (Table 33) indicate that the incubation temperature did not affect the larval susceptibility to infection, but there was a marked effect upon incubation periods (Table 34) and survival times (Table 35). Incubation periods were roughly 2.5 to 5.0 times longer and survival times 1.2 to 2.4 times longer, at 5° than at 20°.

The temperature also affected the rate of development of the insect. The time for larvae to pupate (among those which did not become infected with TIV) was increased at 5°, compared with 20°, by a factor similar to the increase in incubation period. Two adults which emerged at 5° did so 23 and 25 days after pupating, whereas the time from pupation to the emergence of the adult at 20° is about 7 days.

b) Comparison of TIV infection in T. oleracea at five different temperatures

Ten larvae, each of which had been injected with approximately 10^2 LD50 units TIV, were incubated at each of the following temperatures: 5, 10, 15, 20 and 25°. The numbers of insects in which a TIV infection

Table 33. Numbers of insects becoming infected with TIV at 5° and 20°.

Dilution of TIV	5°		20°	
	No. becoming infected *	Percentage becoming infected	No. becoming infected *	Percentage becoming infected
10^{-1}	2/2	100	3/3	100
10^{-2}	3/3	100	2/2	100
10^{-3}	2/2	100	3/3	100
10^{-4}	3/3	100	1/1	100
10^{-5}	2/3	67	2/3	67
10^{-6}	0/2	0	0/3	0
10^{-7}	0/2	0	0/2	0
10^{-8}	0/3	0	0/2	0

* Each result is expressed as the number of insects developing iridescence, followed by the number injected less any which died without iridescence before the day of the mean incubation period for that dose-group.

Table 34. Incubation periods (in days) of TIV-infected larvae at 5° and 20°

Dilution of TIV	5°		20°		$\frac{\bar{I}_5}{\bar{I}_{20}}$
	Incubation periods	Mean ($\bar{I}_5$)	Incubation periods	Mean ($\bar{I}_{20}$)	
10^{-1}	21, 27	24.0	6, 6, 7	6.3	3.8
10^{-2}	21, 31.5, 34	28.8	3.5, 7	5.3	5.4
10^{-3}	22, 23	22.5	7, 8, *	7.5	3.0
10^{-4}	24.5, 27, 28	26.5	10.5	10.5	2.5
10^{-5}	24.5, 29	26.8	8, 13	10.5	2.6

* Infection in the third insect of this dose-group was not detected until the pupal stage, therefore an incubation period was not recorded for it.

Table 35. Survival times (in days) of TIV-infected larvae at 5° and 20°

Dilution of TIV	5°		20°		$\frac{\bar{S}_5}{\bar{S}_{20}}$
	Survival times	Mean ($\bar{S}_5$)	Survival times	Mean ($\bar{S}_{20}$)	
10^{-1}	31.5, 45.5	38.5	14, 24.5, 27	21.8	1.8
10^{-2}	22, 56.5, 75	51.2	21, 27	24.0	2.1
10^{-3}	63.5, 72	67.8	24.5, 31.5 *	28.0	2.4
10^{-4}	31.5, 51, 106	62.8	54	54.0	1.2
10^{-5}	38, 45.5	41.8	10.5, 28	19.3	2.2

* Infection in the third insect of this dose-group was not detected until the pupal stage, therefore a survival time was not recorded for it.

was detected, either by the appearance of iridescence or by the latex test, are shown in Table 36. The results suggest that the larvae were less susceptible to infection with TIV at temperatures below 10° and above 15°, but virtually all those larvae which died without iridescence did so within a few days of being inoculated, so these differences may not be significant. It appeared that the larvae were unsuited to a temperature of 25°, as the larvae hardly fed at all, and died comparatively rapidly at this temperature.

The effects of temperature upon the incubation periods and survival times are indicated in Figs. 22 and 23 respectively. The most marked effect was at 5°, where both incubation periods and survival times were greatly prolonged. NPV infections of leatherjackets appear to be similarly affected by temperature. It was reported (Annual Report of Insect Pathology Unit, Oxford, 1969), that low temperatures considerably delayed the appearance of symptoms in NPV-infected T. paludosa larvae, and Smith (1955) noted that symptoms appeared in about 14 days during spring and summer, but took a month or more in winter.

It was shown that TIV can replicate throughout the temperature range 5 - 25°, and that its optimum lies between 15 and 25°. Gravell and Granoff, (1970) suggested that viruses of poikilotherms replicate within most of their permissive temperature ranges at a rate proportional to the incubation temperature, the rate being fastest slightly below the upper temperature limit. This appears to be the case with TIV, although the rate of virus replication at temperatures below the optimum is not directly proportional to the temperature. The Arrhenius plot (Fig. 22b)

Table 36. Numbers of insects becoming infected with TIV at different temperatures.

Temperature (°C)	No. becoming infected *	Percentage becoming infected
5	6/7	85.7
10	8/8	100.0
15	10/10	100.0
20	8/9	89.9
25	6/10	60.0

* Each result is expressed as the number of insects which were TIV-positive, followed by the number injected less any which died and were TIV-negative up to two days before iridescence first began to appear in that treatment-group.

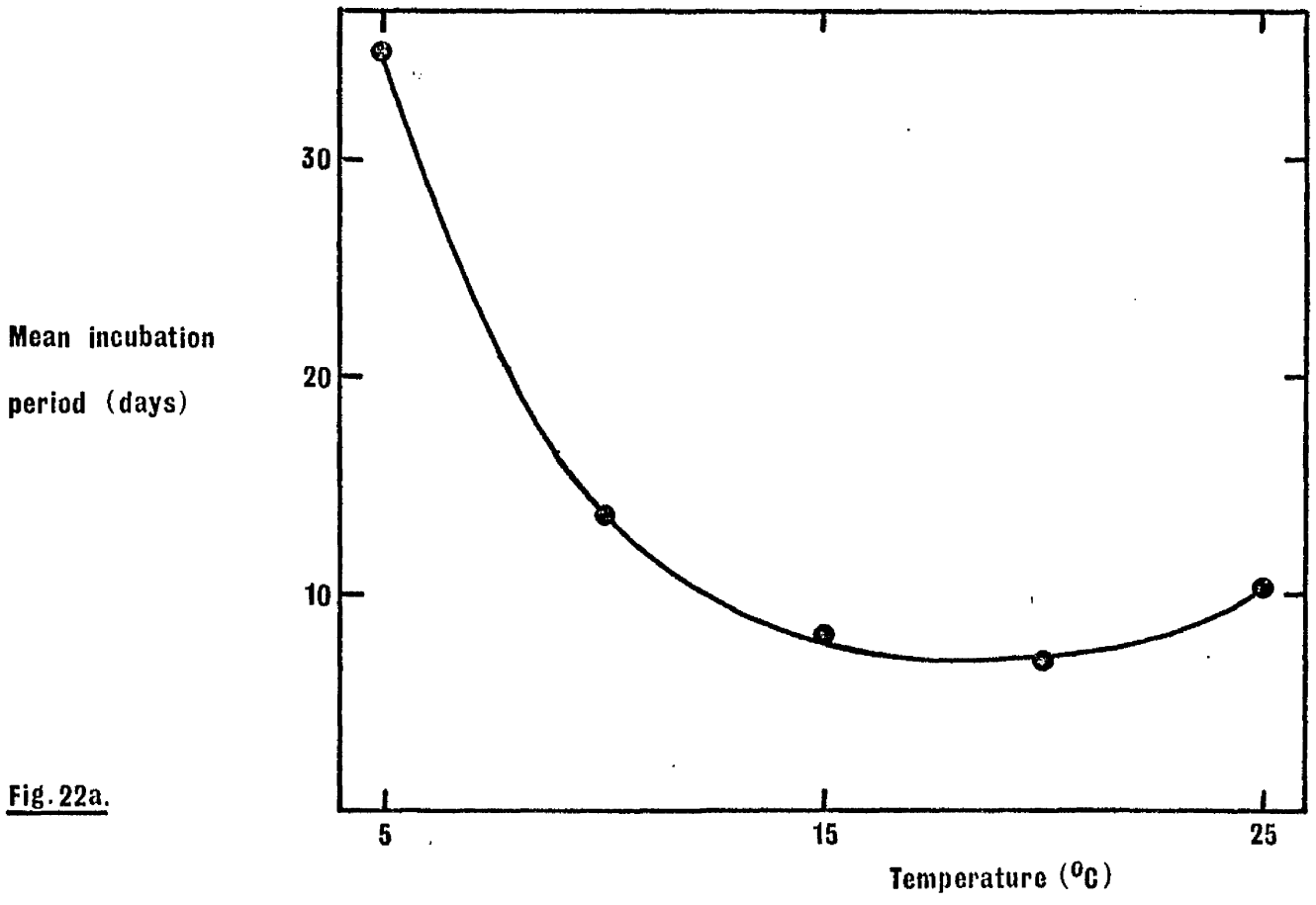


Fig. 22a.

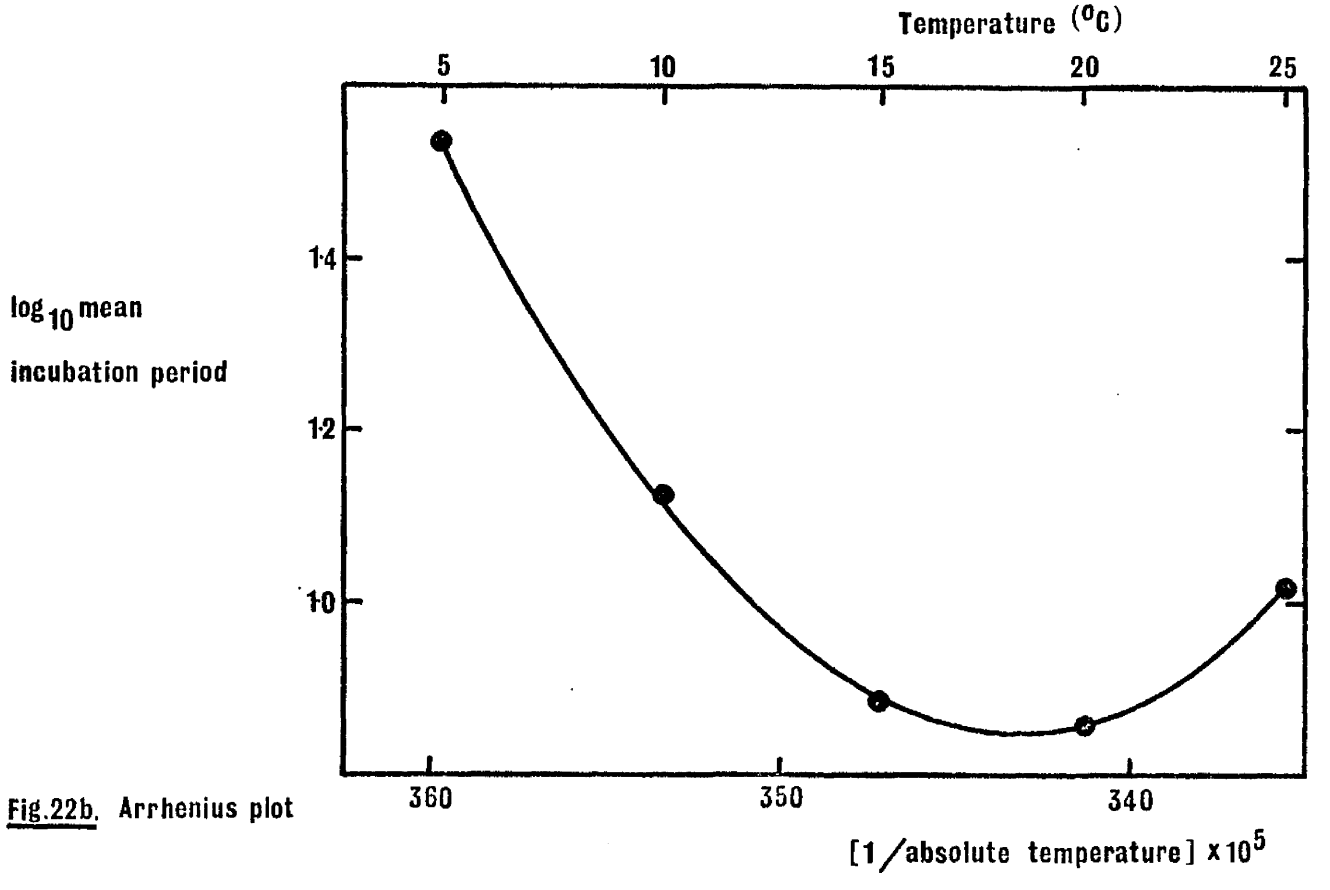


Fig. 22b. Arrhenius plot

Fig. 22. Effect of temperature upon incubation period in TIV-infected T. oleracea larvae.

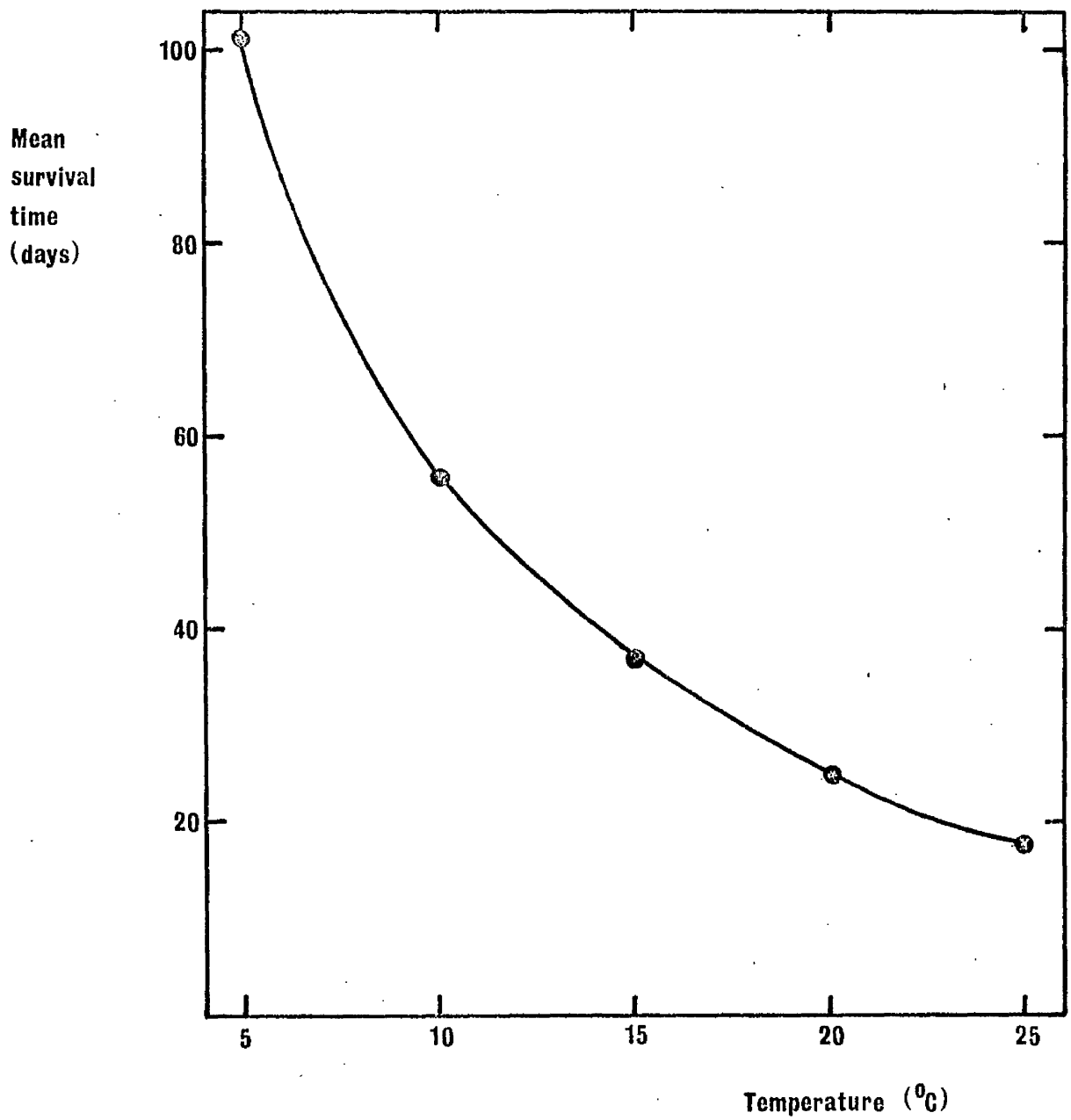


Fig.23. Effect of temperature upon survival time in TIV-infected T.oleracea larvae.

is not a straight line, which suggests that the rate of replication at temperatures below the optimum is limited by more than one enzyme reaction.

Experiment 22. Effects of fluctuating temperatures on TIV infection in leatherjackets.

Introduction

It has been shown (Experiment 21) that temperature has a marked effect upon TIV infection in the leatherjacket. In the natural environment of the insect the temperature is not constant, but is continually varying, with daily fluctuations superimposed upon seasonal fluctuations.

The temperatures to which leatherjackets are exposed also depend on their position in the soil, as the temperature varies with depth. Milne, Laughlin and Coggins (1965) did not find T. paludosa larvae below a depth of 2.5 cm in grassland, but Sellke (1936) found the same species at 10 cm. Stewart (1969) suggested that the larvae do not move deeper into the soil in winter. Leatherjackets feed at the surface in mild, damp weather during the hours of darkness (Dawson, 1932; Ahmed, 1968), therefore they are exposed to surface temperatures, which usually drop below soil temperatures at night. However, leatherjackets do not feed when temperatures are low (Rennie, 1927), so they are exposed to surface temperatures less in winter than in summer.

There are conflicting reports concerning the effects of low temperatures on leatherjackets. Rennie (1927) was of the opinion that frosts have little effect upon leatherjackets, but White (1963) and Ricou (1967) have stated that severe frosts cause reductions in numbers. Insects do not freeze until the temperature is below the freezing-point of their tissues, which undergo supercooling (Salt, 1961), so damage due to freezing will not occur until the temperature is well below 0^{o}.

Larvae inoculated with TIV were exposed to field temperatures in order to study the effects of fluctuating temperatures upon the course of the disease.

Methods

A TIV suspension in ISA was prepared and stored at -16° in 1 ml amounts. At 4 different times of the year decimal dilutions to 10^{-8} of this suspension were prepared, and each dilution was injected into 10 larvae. In addition, 10 larvae were inoculated with the suspension per os (see Experiment 14) and 10 were injected with ISA as controls. Each dose-group was split into 2 groups of 5 larvae, and these were placed in separate plastic containers. The containers were kept out-of-doors in a hole, approximately 10 cm deep, covered with a wooden board.

The larvae in one container were examined daily, while those in the other were examined twice-weekly. These differences in frequency of inspection were designed to detect any effect caused by the difference in temperature which the insects encountered when they were brought into the laboratory for examination, which lasted for about 20 min on each occasion.

The soil temperature beside the containers was measured daily with a maximum and minimum thermometer at a depth of approximately 8 cm. An additional group of 10 larvae was injected with the undiluted TIV suspension and incubated at 20° as a control. All insects which died without developing iridescence were tested for the presence of TIV by the latex test.

Results and Discussion

Numbers of insects becoming infected

There was no evidence that the susceptibility of the larvae to infection with injected TIV was affected by temperature. Similar numbers of insects became infected after each series of inoculations throughout the year (Table 37), and the LD50s did not differ significantly. These results are in agreement with those of Experiment 21, where the LD50 was identical at 20° and at 5°.

The results suggest that lower temperatures may increase the probability of a larva becoming infected *per os*. Further experiments will be necessary to determine whether this is so.

Effect of temperature on the course of the disease

The temperatures throughout the course of the experiment are indicated in Fig. 24, together with the rates of appearance of iridescence and the death rates for the larvae injected with the undiluted TIV suspension. The winter 1970-71 was exceptionally mild, and the insects were rarely exposed to temperatures below 0°. During the colder months of the year, both incubation periods and survival times were greatly prolonged compared with the warmer months (Tables 38 and 39). The ratio time at fluctuating temperature/time at 20° was used in an attempt to compensate for variation between batches of larvae, and for loss of infectivity of the TIV preparation during the course of the experiment (see Experiment 23).

The results are in agreement with those obtained at constant temperatures (Experiment 21) in that there was an increase in the rate of TIV replication with increasing temperature up to 20°. It can be concluded

Table 37. Numbers of insects becoming infected with TIV at different times of the year

Treatment-group	Date of inoculation			
	2.5.1970	7.7.1970	21.9.1970	29.11.1970
10^{0}	9/9	10/10	10/10	8/8
10^{-1}	10/10	9/10	10/10	6/6
10^{-2}	9/9	9/10	9/9	6/7
10^{-3}	9/10	6/9	7/9	5/7
10^{-4}	9/10	3/10	6/10	3/5
10^{-5}	4/10	4/10	6/8	3/8
10^{-6}	3/9	1/9	3/9	0/8
10^{-7}	0/8	1/10	0/9	1/9
10^{-8}	0/8	0/10	0/10	0/8
per os	3/10	1/9	0/10	5/9
ISA	0/9	0/10	0/10	0/6
10^{0} (incubated at 20^{0})	10/10	10/10	8/8	10/10
LD50*	$10^{-5.0}$	$10^{-3.9}$	$10^{-4.9}$	$10^{-4.2}$
S.E. of LD50	$10^{0.27}$	$10^{0.34}$	$10^{0.20}$	$10^{0.37}$

Each result is expressed as the number of insects which were TIV-positive, followed by the number inoculated less any which died and were TIV-negative up to 2 days before iridescence first began to appear in that treatment-group.

* The LD50 values were determined by probit analysis.

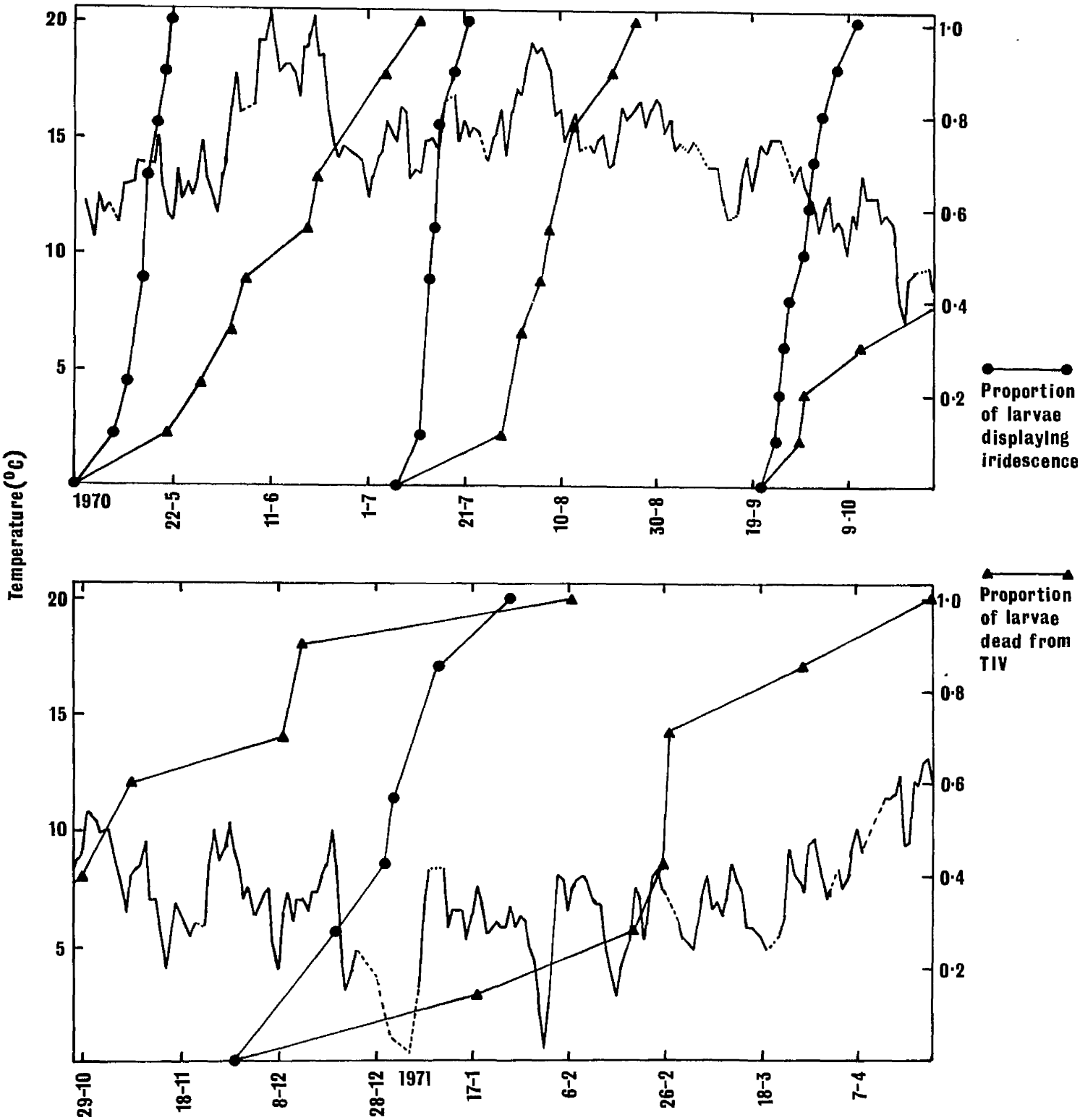


Fig.24. Effect of field temperatures upon incubation period and survival time in TIV-infected T.oleracea larvae.

The results are those for the larvae injected with the undiluted TIV suspension.
Each temperature is the mean of the maximum and minimum soil temperatures measured at a depth of approximately 8cm. Most temperatures were read daily, but a longer interval, indicated by dotted lines, elapsed between a few of the readings.

Table 38. Effect of fluctuating temperatures upon incubation periods.

Date of inoculation	2.5.1970	7.7.1970	21.9.1970	29.11.1970
Mean temperature *1	12.5°	15.1°	12.7°	6.3°
$\frac{\bar{I}_{ft}}{\bar{I}_{20}}$ *2	2.65	1.43	1.70	4.19

*1 The mean of the daily mean temperatures over the period elapsing between the day of inoculation and the day when the last larva developed iridescence.

*2 $\bar{I}_{ft}$ = mean incubation period at fluctuating temperature.

$\bar{I}_{20}$ = mean incubation period at 20°.

Table 39. Effect of fluctuating temperatures upon survival times.

Date of inoculation	2.5.1970	7.7.1970	21.9.1970	29.11.1970
Mean temperature *1	14.9°	15.7°	8.0°	6.87°
$\frac{\bar{S}_{ft}}{\bar{S}_{20}}$ *2	1.80	1.24	2.31	3.65

*1 The mean of the daily mean temperatures over the period elapsing between the day of inoculation and the day when the last larva died.

*2 $\bar{S}_{ft}$ = mean survival time at fluctuating temperature.

$\bar{S}_{20}$ = mean survival time at 20°.

that field temperatures, which vary over the year from below 0° to above 20°, have a marked effect upon the rate of TIV replication in leatherjackets, with the rate being relatively rapid in autumn, slow during the winter and increasing when temperatures begin to rise in the spring. As the first 2 larval instars of T. paludosa and T. oleracea are completed in the autumn it is probable that these larvae develop iridescence if they become infected with TIV, although iridescence in first- and second-instar larvae has not yet been reported in the field.

Larvae may become infected in the late autumn or early winter and not develop iridescence until the late winter or early spring. Examples of such long incubation periods were observed by Newbold, J.W. (personal communication), who collected T. paludosa larvae from the field in early December 1965, and early January 1966. He kept them in pots out-of-doors, and in March 1966 observed that some of them developed iridescence. It is probable that these larvae were infected with TIV when they were collected from the field, but did not develop iridescence for 2 - 3 months because of the low temperatures prevailing during the winter.

Effects of temperature on T. oleracea

As well as affecting the course of the disease, temperature also had an effect upon the insect. During the warmer months, many of the larvae which did not become infected with TIV, e.g. those injected with high dilutions of TIV or with ISA, pupated and gave rise to adults. In the winter months, however, very few larvae pupated, and of those which did, most died in the pupal stage; the majority of the larvae which did not become infected eventually died. Although the larvae were exposed to

natural variations in temperature, the situation was nevertheless artificial as they had been reared at room temperature (approximately 20°) until they were inoculated in the fourth instar.

Temperature apparently affected the rate of development of the insect. At higher temperatures larvae pupated and gave rise to adults more quickly than at lower temperatures.

Differences between daily-inspected and twice-weekly inspected groups

There were no significant differences between those larvae examined daily and those examined twice-weekly with regard to numbers becoming infected, incubation period or survival time, therefore there was no evidence that bringing the insects into the laboratory for inspection had affected either their susceptibility to infection or the rate of virus replication.

There were, however, significant differences between the daily- and twice-weekly-inspected groups in the numbers of larvae which died without evidence of a TIV infection. Many of these died within a short time of inoculation. For example, of the larvae inoculated on 2.5.1970, 12 and 26 died from 'unknown causes' in the daily- and twice-weekly-inspected groups respectively. A χ^2 test showed that this difference is significant ($p < 0.05$). The reason for these differences between the 2 groups may be that it was possible to maintain the moisture in the daily-inspected dishes at a more constant level than in the twice-weekly-inspected dishes. Some of the larvae in the latter dishes may have died from an excess or a deficiency of moisture.

Experiment 22a. A strain of T. oleracea with an altered response to TIV

Introduction

Variation in susceptibility to parasites between host individuals and host populations is common, and several examples have been reported among insects. Martignoni and Schmid (1961) found that 2 populations of Phryganidia californica differed in susceptibility to NPV, and Stairs (1965a) found that individual Galleria mellonella larvae varied in susceptibility to NPV. Three stocks of Pieris brassicae were found to differ in susceptibility to granulosis virus by David and Gardiner (1965b), and the difference between 2 of the stocks was shown to persist through about 36 generations. Harvey and Howell (1965) obtained a strain of the house fly, Musca domestica (Linnaeus), with increased resistance to Bacillus thuringiensis by breeding from the survivors of larvae treated with the bacterium.

During the present investigations, records were kept of the progeny groups of most of the larvae used for experiments. When analysing the results for the first group of larvae in Experiment 22 it appeared that the incubation periods and survival times of one progeny group (17E) were longer than those of the others. By the time this was realised the insects had passed through 2 more generations. A progeny group of larvae in the second filial generation descended from the 17E insects (21G) were compared with other progeny groups in the same generation for any possible resistance to TIV.

Results

The incubation periods and survival times for the larvae which became infected in Experiment 22 are given in Tables 40 - 41, and the times from inoculation to pupation of the larvae which did not become infected are given in Table 42. Approximately half of the larvae for this experiment had been taken from group 17E, and the rest were from 5 other progeny groups. Significant differences in incubation period and survival time between 17E and the other larvae were found in some treatment groups; the lack of significance in the other treatment groups may be due to the small numbers of insects involved. A t test indicated that there was a significant difference ($p < 0.001$) between the times to pupation of the 17E larvae and those of the other progeny groups.

There was no evidence that the 17E larvae were less susceptible to infection with TIV than the other progeny groups, in fact the results suggested that the converse might be true (Table 43), but the numbers of insects are too small to test whether this is significant.

These results suggested that the 17E insects developed more slowly than the other progeny groups, and that TIV replicated in them more slowly. It was considered possible that this was a hereditary characteristic as all the progeny groups had been kept under similar environmental conditions. By the time the above results were examined the next generation had passed; there was 1 progeny group (21G) in the succeeding generation descended from 17E (see Fig. 26).

Table 40. Incubation periods (in days) of 17E larvae and other progeny groups.

Treatment group	Progeny group 17E	Mean	Other progeny groups	Mean	t test
10^{0}	14, 15, 19, 22	17.5	8, 11, 14, 15, 17	13.0	*
10^{-1}	11.5, 15, 15, 16, 18	15.1	10, 11.5, 11.5, 11.5, 16	12.1	NS
10^{-2}	15, 18, 22	18.3	11, 11.5, 12, 15, 22	14.3	NS
10^{-3}	18, 18.5, 20, 43	24.9	11.5, 13, 13, 18	13.9	*
10^{-4}	20, 20, 22, 22	21.0	11, 11.5, 13, 15, 22	14.5	*
10^{-5}	21, 21	21.0	17, 24	20.5	NS
10^{-6}	15	15.0	22	22.0	NS
per os	15, 22	18.5	13	13.0	NS
10^{0} (incubated at 20^{0})	8, 9, 10,11	9.5	6, 7, 8, 9, 9, 12	8.5	NS

The results of the t test are given as either * (significant difference, $p < 0.05$)

or NS (no significant difference).

Table 41. Survival times (in days) of 17E larvae and other progeny groups.

Treatment group	Progeny group 17E	Mean	Other progeny groups	Mean	t test
10^0	26, 50, 64, 71	52.8	19, 32, 35.5, 43, 64	39.7	NS
10^{-1}	54, 57, 59, 60.5	57.6	18.5, 22, 25.5, 38	26.0	*
10^{-2}	25.5, 45, 57	42.5	15, 24, 25.5, 25.5	22.5	*
10^{-3}	48, 51, 57, 64	55.0	21, 22, 35, 57	33.8	NS
10^{-4}	31.5, 43, 62, 78	53.6	15, 18.5, 19, 78	32.6	*
10^{-5}	52, 88	70.0	29, 32	30.5	NS
10^{-6}	50	50.0	28.5	28.5	NS
per os	25.5, 48	36.8	44	44.0	NS
10^0 (incubated at 20^0)	26, 27, 30, 31	28.5	14, 15, 23, 28, 28, 31	23.2	NS

The results of the t test are given as either * (significant difference, $p < 0.05$) or NS (no significant difference).

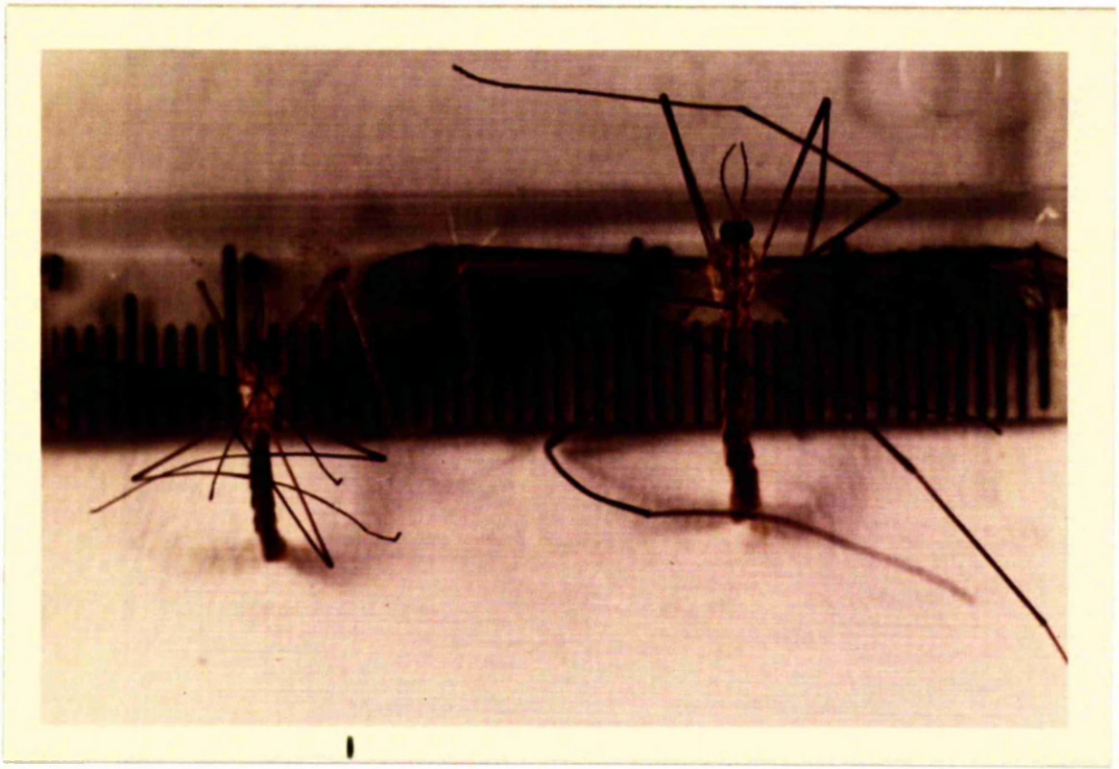
Table 42. Times from inoculation to pupation (in days) of 17E larvae and other progeny groups.

Treatment group	Progeny group 17E	Mean	Other progeny groups	Mean
10^{-5}	71	71.0	43	43.0
10^{-6}	76	76.0	20	20.0
10^{-7}	67	67.0	10, 11.5	10.8
10^{-8}	59, 83	71.0	15, 18	16.5
per os	44, 64	54.0	51	51.0
ISA	50, 57, 59	55.3	13	13.0

Table 43. Numbers of 17E and other progeny groups becoming infected with TIV.

Treatment group	Progeny group 17E		Other progeny groups	
		Percentage		Percentage
10^{0}	4/4	100	5/5	100
10^{-1}	5/5	100	5/5	100
10^{-2}	3/3	100	6/6	100
10^{-3}	4/4	100	5/6	83
10^{-4}	4/4	100	5/6	83
10^{-5}	2/4	50	2/6	33
10^{-6}	1/4	25	2/5	40
10^{-7}	0/3	0	0/5	0
per os	2/4	50	1/6	17
10^{0} (incubated at 20^{0})	4/4	100	6/6	100

Each result is expressed as the number of insects which were TIV-positive, followed by the number inoculated less any which died and were TIV-negative up to two days before iridescence first began to appear in that treatment-group.



21G male normal male

Fig. 25. Small and normal-sized T. oleracea male adults

Scale : mm.

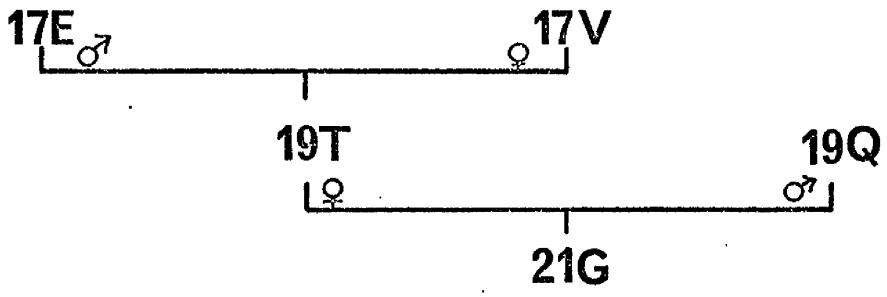


Fig. 26. Family tree of progeny group 21G.

Unfortunately very few of the 21G larvae survived to the fourth instar. To compare the reaction to TIV of 21G with that of other progeny groups, 6 21G larvae and 10 larvae from each of 5 other progeny groups were injected with equal doses of TIV. The mean incubation period and the survival time for the 21G larvae were both longer than for any of the other progeny groups (Table 44); the t test indicates a significant difference ($p < 0.05$) for the incubation periods, but not for the survival times.

The remaining 21G larvae which were not injected with TIV appeared to develop more slowly than the other progeny groups, and only 2 of them survived to pupate. One of these pupae died, and the other gave rise to a male fly which was smaller than the normal male (Fig. 25). Its wing span, abdomen length and overall length (from the tips of the antennae to the tip of the abdomen) were measured, and compared with those for a sample of 10 males from other progeny groups (Table 45). The 21G male was found to be approximately 2/3 normal size.

Table 44. Mean incubation periods and survival times for 21G and five other progeny groups inoculated with TIV.

Progeny group	Mean incubation period (days)	Mean survival time (days)
21G	8.2	31.2
21K	6.3	26.4
21L	5.8	24.0
21M	7.4	26.6
21N	6.4	24.3
21O	5.8	24.7

Table 45. Comparison of size of the 21G male adult with a sample of ten other males (all lengths in cm).

	Wing span	Abdomen length	Overall length
Progeny group 21G	2.4	0.9	1.4
Other progeny groups (range)	3.5 - 3.9	1.2 - 1.4	2.0 - 2.5
Other progeny groups (mean)	3.7	1.2	2.2
Ratio of 21G length/mean length of other progeny groups	0.65	0.74	0.64

This male was observed to show an interest in female crane flies, but not to mate successfully. On separate occasions it was confined with 2 females, both of which laid eggs which did not hatch. The male did not survive for long, dying between 1 and 2 days after emergence, and thus the strain descended from 17E was lost.

Discussion

Both the progeny group 17E and its descendants 21G had a slower than average rate of development, and the results suggest that TIV replicated in them more slowly, although there was no evidence that 17E was any more resistant to infection with TIV. Both of these phenomena could possibly be explained by a slower metabolic rate in the insects, and it appears that this factor may have been inherited by the 21G insects from 17E. Differences in rate of development between different progeny groups of insects were observed in Experiment 7. Bergold (1953) observed that polyhedrosis viruses develop faster in more vigorous insects.

An insect strain such as the 17E progeny group would be disadvantageous in the field from the point of view of biological control with TIV because the time to kill the insect is prolonged. It is unlikely, however, that such a strain would survive for long because of its reduced vigour.

E. EFFECTS OF ENVIRONMENTAL FACTORS UPON TIV

Introduction

Insect viruses in general tend to possess a high degree of stability outside the host. This fact was noted by Bergold (1952), who stated that many can be stored dry or wet for several years without much change in infectivity, and by Rivers (1966b), who observed that the protein crystals within which many insect viruses are occluded, confer protection against most virucidal factors, including ultra-violet irradiation. A preparation of Colias eurytheme NPV was still infective after it had been smeared on glass slides and exposed to the weather for 2 years (Thompson and Steinhaus, 1950). The present investigations were concerned mainly with the long-term effects of temperature upon TIV; the effects of pH on the virus were also studied.

Experiment 23. The effect of temperature upon TIV

Introduction

Several insect viruses have been shown to be stable at different temperatures. A preparation of Bombyx mori NPV was found to be still infective after storage at 4° for 20 years (Steinhaus, 1960). Neilson and Elgee (1960) stored polyhedrosis virus-infected larval cadavers of the European spruce sawfly at 4.5° and observed a slight drop in infectivity over the first 9 years, then a more rapid drop, with total inactivation occurring during the twelfth year. Shapiro and Ignoffo (1969), studying free virions of Heliothis zea NPV, observed 16-fold, 100-fold and 600-fold decreases in infectivity after 225 days at 5°, 37° and 50°

respectively. Cunningham (1970) found that a preparation of the NPV of the eastern hemlock looper, Lambdina fiscellaria fiscellaria (Guenee), which had been stored at 4° for 6 years, had decreased in infectivity approximately 200-fold. Sigma virus of Drosophila is one of the less stable insect viruses, having a half-life of 8.0 h at 25° (Seecof, 1969).

Both TIV and SIV appear to be fairly stable viruses. Smith, K.M. (personal communication to Thomas, 1961) stated that TIV was physically and biologically stable when stored in a cold room, but Wrigley (1970) found that TIV particles which had been stored in water for about 2 months at 2° had begun to disintegrate into triangular, pentagonal and linear fragments. He also observed similar disintegration of SIV particles after prolonged storage in water at 4° (Wrigley, 1969), and Day and Gilbert (1967) found that SIV gradually lost infectivity when stored in a refrigerator. Linley and Neilsen (1968b) found that the MIV from Aedes taeniorhynchus lost infectivity rapidly at 27°; all infectivity was lost after storage in seawater for 20 days and in artesian water for 10 days.

In order to investigate the stability of TIV, samples of the virus were stored at 3 different temperatures, and assayed at regular intervals over a period of 1 year.

Methods

A purified sample of TIV was suspended in deionised water, assayed by the response time method, and then split into 1 ml portions. Some of these were stored at 20°, some at 5° and some were stored in a deep-freeze (approximately -16°). At about 10-week intervals over a period of 1 year a portion of TIV was removed from each batch, and its infectivity was assayed by the response time method.

Results and Discussion

The results, which are summarised in Fig. 27, indicate that the TIV suspensions at all 3 temperatures lost infectivity over a period of 12 months. The loss of infectivity at 20° was about 10^{5}-fold, while at 5° and -16° it was about 10^{3} - 10^{4}-fold. No significant difference in infectivity was detected between the virus stored at 5° and that at -16°.

The results can only be considered as approximate estimates of the virus titres because of the high variability of the response times in the larvae used for the assays. The results of an assay performed on day 154 are not included in Fig. 27 as they are highly anomalous, with an apparent increase in titre at each storage temperature. Such variation suggests some difference between the larvae inoculated at day 84 and those inoculated at day 154, resulting in shorter response times in the latter case. The results for day 154 (in $\log_{10}$ LD50 units/ml) were: 20°, 6.25; 5°, 7.21; -16°, 5.28.

It appears, then, that TIV is a fairly stable virus. Thus, for convenience in the laboratory, TIV can be prepared in bulk and stored in a refrigerator or a deep-freeze. The preparation should contain infective virus for at least a year, but the titre will gradually decline so that it will be necessary to assay the preparation at the time it is used. Freeze-drying may be worthy of investigation as a technique for the long-term storage of TIV.

In this experiment the virus was suspended in deionised water, so the results do not indicate how TIV might survive in the soil, on plant surfaces or in infected cadavers.

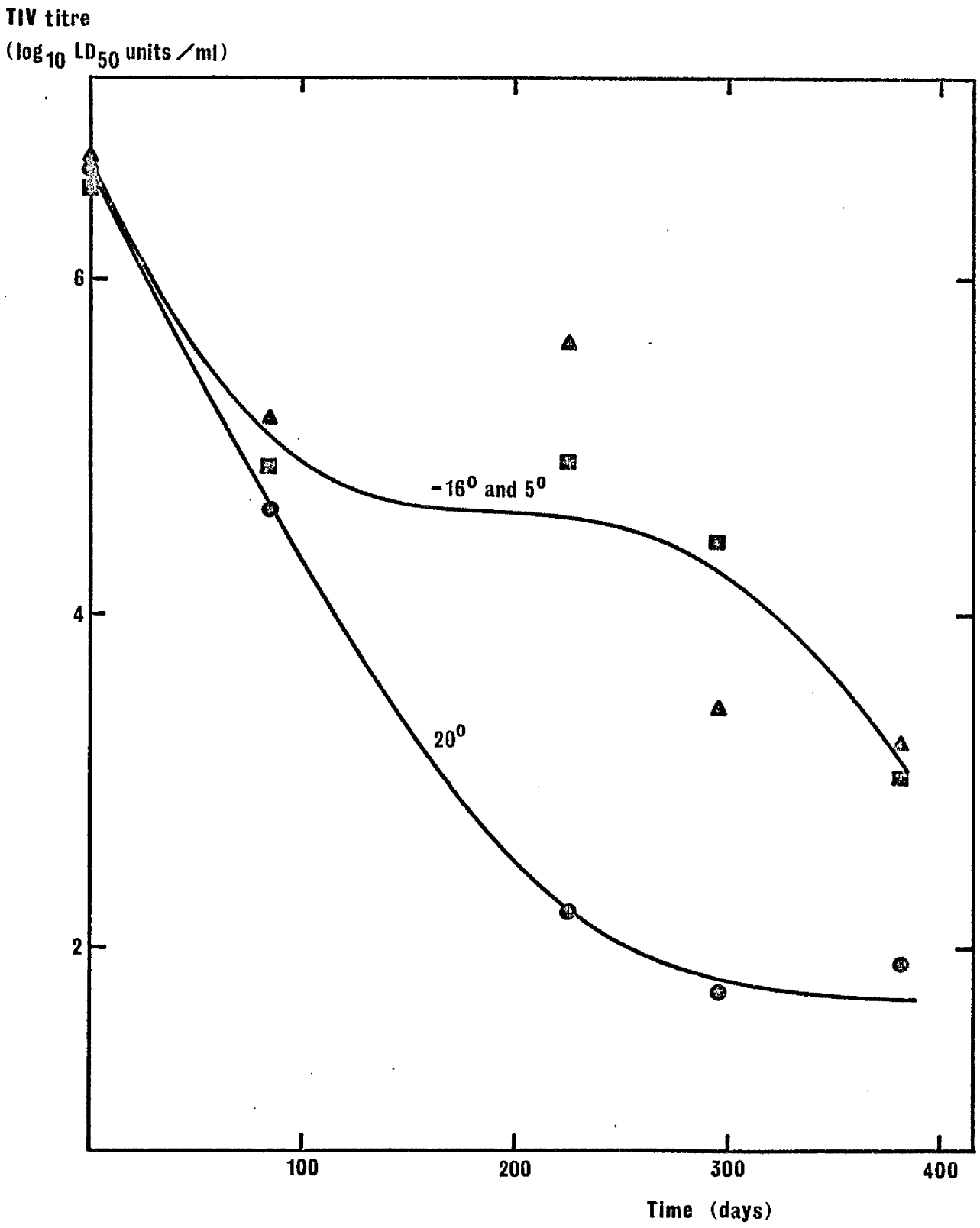


Fig.27. Assays of TIV suspensions stored at three different temperatures.

● —— ● 20°

■ —— ■ 5°

▲ —— ▲ -16°

Experiment 24. Ultraviolet spectrophotometry of TIV at different pHs

Introduction

Smith and Hills (1959) reported that the capsid of the TIV particle is highly resistant to changes in pH, but Glitz et al (1968) noted a partial denaturation of the virus in the pH range 9.5 - 12.0, as judged by the formation of a sticky and less iridescent pellet after centrifugation. Virions were observed to become distorted at pH $>$ 10.0, but the DNA and protein remained associated up to at least pH 12.0.

The effect of pH upon TIV was studied by determining the UV absorption spectra over the range pH 1.0 - 12.0.

Methods

A TIV preparation was purified by 5 cycles of low-/high-speed centrifugation followed by centrifugation in a sucrose gradient. It was washed with water, then equal amounts were suspended in buffer solutions at 12 different pHs (Table 46). The UV spectra of these suspensions were determined as described in Materials and Methods (p. 27).

Table 46. Buffer solutions used to prepare TIV suspensions.

pH values	Buffer
1.0, 2.0, 3.0, 4.0, 5.0	acetate + HCl
6.0, 7.0, 8.0	Na_2HPO_4 + NaH_2PO_4
9.0, 10.0, 11.0, 12.0	borate + NaOH

Results and Discussion

The results are shown in Fig. 28. Large corrections for light scattering were necessary except at pH 1.0 and 12.0, where presumably some degradation of the virus occurred. The UV spectra of the TIV suspensions at pH values around 7.0 appear to be similar to that published by Glitz et al (1968) for TIV and SIV, and that published by Matta (1970) for MIV. Small differences between these published spectra and those in Fig. 28 may be due to the fact that these workers corrected their results according to the method of Bonhoeffer and Schachman (1960), whereas the method of Kleczkowski (1968) was used for the present results.

The effect of pH on UV absorption at 260 nm and 280 nm, which are the regions of maximum absorption for nucleic acids and proteins respectively, is indicated in Fig. 29. There appears to be little effect on absorption, except at extreme pH values, which suggests that the virus particles are fairly stable throughout a wide range of pH. It would be interesting to determine the effect of pH on the infectivity of the virus. It may be that the infectivity of TIV is affected by the pH in one of the sections of the larval gut, where the pH varies between about 5.8 and >8.5 (Experiment 15); this may possibly account for the low infectivity of the virus when it is ingested by the insect, compared to other routes of infection.

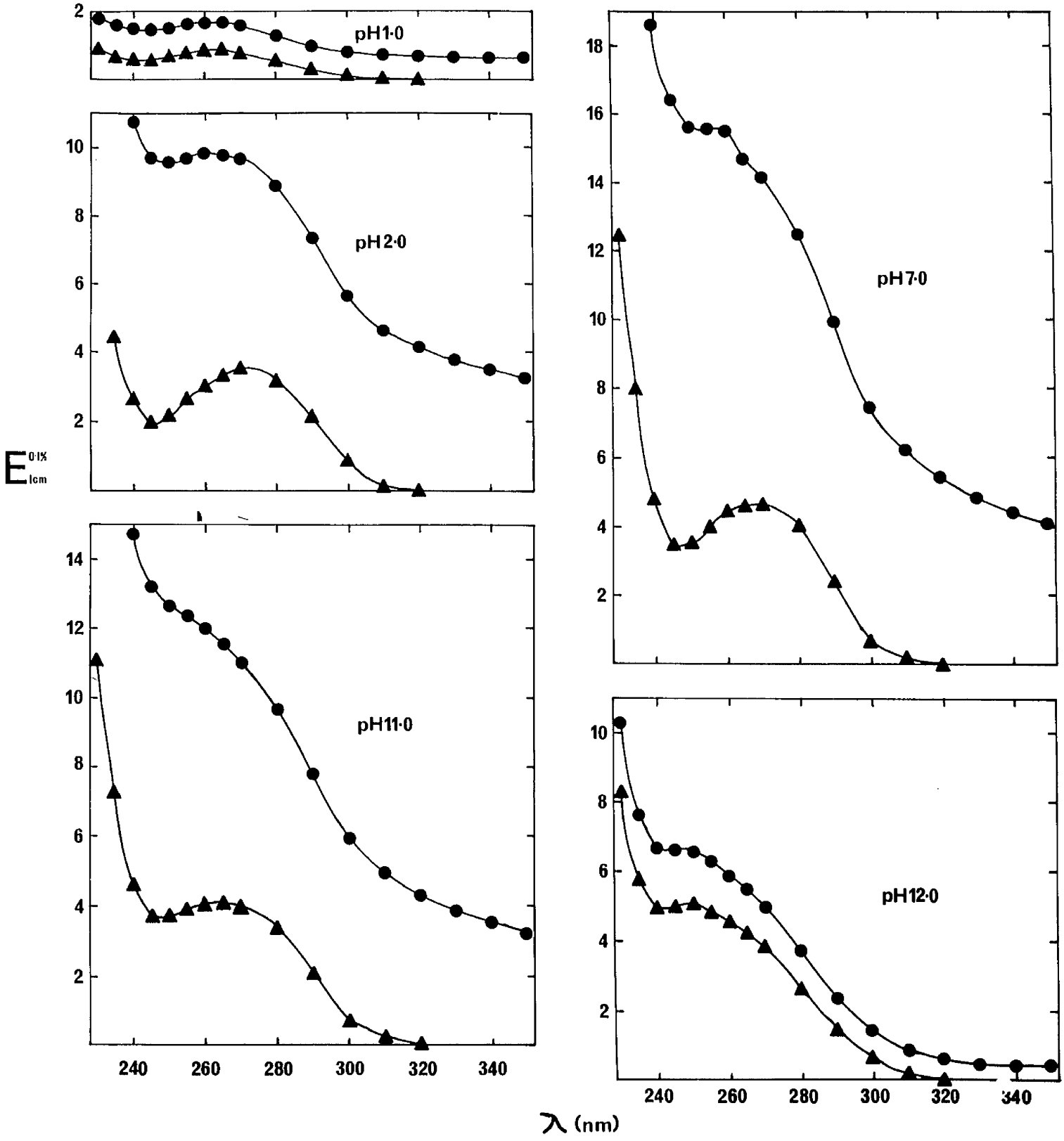


Fig.28. UV absorption spectra of TIV suspensions at different pHs.

The spectra at pH 3·0–6·0 and pH 8·0–10·0 resembled the spectrum at pH 7·0.

●——● uncorrected values

▲——▲ values corrected for light scattering

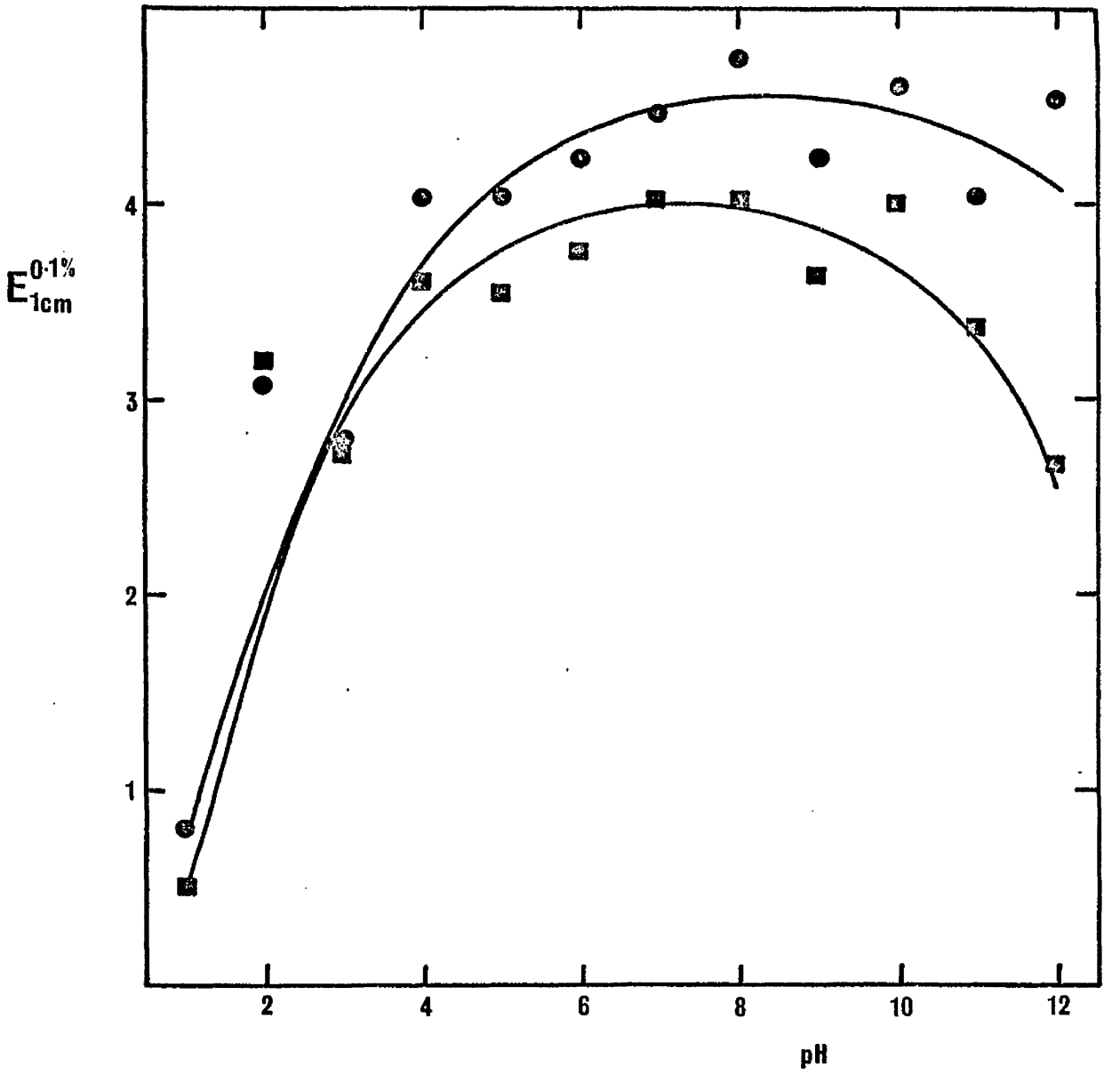


Fig.29. The effect of pH upon UV absorption by TIV suspensions at 260 nm (●——●) and 280 nm (■——■).

Values corrected for light scattering.

GENERAL DISCUSSION

TIV infection in T. oleracea

The experiments described in this report have demonstrated that all stages in the life-cycle of T. oleracea, except the embryo, are susceptible to infection with TIV. Iridescence resulting from TIV infection was observed in all 4 larval instars, pupae, and adults of both sexes. This situation contrasts with HIV infections in Aedes taeniorhynchus (Linley and Neilsen, 1968a), where iridescence rarely appears before the fourth larval instar, even if the insects have been infected from the egg.

In many cases the larvae died from a TIV infection in the instar in which they were inoculated, e.g. in Experiment 16 (main experiment) all the deaths from TIV were in the first instar. However, it was observed on a number of occasions that TIV-infected larvae were able to moult, and a few infected fourth-instars were able to pupate. The situation is probably similar in T. paludosa, although Ahmed (1968) reported that he did not observe any moulting of TIV-infected T. paludosa larvae.

The detection of TIV infection by the latex test in a number of apparently healthy adults (Experiment 19) indicates that sublethal infection with TIV can occur. These adults were derived from larvae and pupae which had been inoculated with TIV.

Transmission of TIV

There is no evidence that TIV is transmitted by insect or nematode parasites of the leatherjacket or that vertical transmission via the gametes occurs (Experiment 20), and it is doubtful whether infection can occur via the spiracles (Experiment 17). Although the faeces of infected larvae

contain TIV (Experiment 9), the amount is not sufficient to serve as a source of infection (Experiment 10). Even newly-hatched larvae, which are possibly more susceptible to infection with TIV than later instars (Experiment 16), did not become infected when exposed to faeces from TIV-infected larvae.

It was shown (Experiments 13 and 14) that large doses of TIV are necessary to cause infection per os. A suitable high concentration of virus is present in infected cadavers, where TIV may constitute 25% of the dry weight (Williams and Smith, 1957).

The evidence suggests that leatherjackets become infected with TIV mainly by feeding on other leatherjackets infected with the virus.

The fact that leatherjackets are cannibalistic has been noted by a number of workers, including Freeman (1966) who frequently found pieces of Tipula body wall in the guts of field populations of leatherjackets, many of which often bore scars. Fighting and cannibalism among T. oleracea larvae was observed on many occasions during the present experiments, and a number of the larvae taken from the rearing pots had black scars, presumably from wounds sustained while fighting with other larvae.

Many leatherjackets survive for long periods after they have become infected with TIV, e.g. in Experiment 9 one TIV-infected larva did not die until 83 days after inoculation. Many TIV-infected larvae could survive until the next generation of eggs had hatched, and serve as a source of inoculum for the newly-hatched larvae. T. paludosa adults are on the wing from July to September (Ahmed, 1968) and T. oleracea adults are on the wing in May and again in August (Freeman, 1967). Under temperature conditions

such as occur during these months, larvae survived for long periods (see Fig. 24), e.g. 50, 64 and 78 days after inoculation with TIV in the fourth instar, which should be more than enough time for the healthy insects to give rise to adults, lay their eggs, and for the eggs to hatch. Ahmed (1968) observed that TIV-infected and NPV-infected T. paludosa larvae may remain alive for up to 13 and 14 weeks, respectively; they may survive until September, by which time the next generation has appeared.

It is suggested, then, that a new generation of leatherjackets becomes infected by feeding on the cadavers of TIV-infected leatherjackets which have survived from the previous generation. As the healthy larvae grow they will feed on their infected brethren, especially when the latter begin to grow weaker as they become moribund. In this way the disease can be transmitted as the larvae grow (Experiment 16), the infection of one larva requiring the death of another.

This suggested mode of transmission for TIV contrasts with that for MIV in Aedes taeniorhynchus (Linley and Nielsen, 1968b) where transovarial transmission of the virus occurs, and the infected larvae do not die until the fourth instar, when they serve as a source of infection for other larvae, which become infected per os and then give rise to infected adults to complete the cycle. Day (1965) found no evidence for transovarial transmission of SIV in Galleria mellonella.

The results of Experiments 21 and 22 suggest that temperatures which favour more rapid development of T. oleracea also favour more rapid replication of TIV; thus both insect and virus develop more rapidly in the warmer months of the year. Laughlin (1967) observed that T. paludosa

larvae grow very fast in the autumn, slowly through the winter, and moderately fast in the spring and early summer. In both T. oleracea and T. paludosa the first 2 instars are completed in the autumn, and in T. paludosa the larvae overwinter in the third instar (Coulson, 1962). It is uncertain whether T. oleracea larvae overwinter in the third or the fourth instar (Stewart, 1969). It is probable then, that TIV replication is fairly rapid in the first 2 instars, slows down in the third instar, and then accelerates again in the fourth instar when the temperature begins to rise.

Experimental leatherjackets in petri dishes usually crawl from beneath the filter paper just before they die from TIV infection, and Ahmed (1968) observed that, in the field, leatherjackets in the later stages of infection with TIV tend to rise to the surface of the soil. These larvae are conspicuous, being blue, green, purple, orange or often almost white in colour; this makes them an easy prey for birds, which have been observed feeding upon TIV-infected larvae at the surface (Newbold, J.N., personal communication).

Hostetter and Biever (1970) found infective virus in the droppings of sparrows which had fed upon NPV-infected cabbage loopers, and suggested that the virus might thus be disseminated by the sparrows. It is unlikely that birds are important in the dissemination of TIV, providing in the first instance that the virus survives in their digestive tracts, because it is unlikely that much virus in the birds' faeces reaches leatherjackets in a concentration high enough to serve as an infective dose.

Epizootics of TIV

TIV is usually found only at low levels in leatherjacket populations. Newbold, J.W. (Personal communication) has sampled between 2,500 and 3,000 fields for leatherjackets; in most cases there was no evidence of TIV infection, and in those cases where TIV infection was found the incidence was below 1%, with 2 exceptions (see below). Sherlock, P.L. (personal communication) found TIV infections in T. paludosa larvae at 2 out of 7 sites, with incidences of 2.3 and 2.4%. Xeros (1956) found the incidence of TIV-infection in leatherjackets to be less than 1% in Cornwall and near Cambridge. Stewart (1969) found only small numbers of leatherjackets infected with TIV; the highest infection rate was 6% of 150 third-instar T. paludosa larvae. The highest rate of TIV infection in leatherjackets observed by Ahmed (1968) was 4.7%.

More severe outbreaks of TIV have been observed by Xeros (1956), who found up to 15% of a field population infected, and Newbold, J.W. (personal communication), who observed one population of T. paludosa larvae with 75% infected with TIV, and another population, which was comprised mainly of T. paludosa larvae, with up to 20% infected.

Although TIV appears to be widespread in distribution, it is rarely found infecting a large proportion of larvae, which, as the virus is usually lethal, in the long term ensures its own survival. It is important to determine the factors which initiate TIV epizootics in order to assess whether they can be induced artificially. Possible factors involved are temperature, population density and the resistance of the population to infection, but it is difficult to draw any conclusions as to which of these may be the most important on the basis of the few reported epizootics of TIV.

The observation of Xeros (1956) of a high percentage of TIV-infected leatherjackets was made in Shropshire in 1954, and those of Newbold, J.M. (personal communication) were made at 2 separate sites in Stirlingshire, one in 1963 and one in 1966. The mean monthly temperatures of these areas in those years are given in Tables 47 - 49, the data being taken from 'The Monthly Report of the Meterological Office, H.M.S.O.' In all 3 cases most of the mean monthly temperatures were below average from January onwards, and it is interesting to note that in the case of the highest percentage infection (75%) it was extremely cold in January and February (mean temperature 0.1°), whereas in the smaller outbreaks (15 and 20%) the mean of the January and February temperatures was higher, although still below average (2.95° and 3.05° respectively).

No correlation can be confirmed because of the small number of observations involved, and in fact the converse of this situation would be expected, as the disease progresses more rapidly with increasing temperature over this range (Experiments 21 and 22).

It may be that the leatherjacket's resistance is lowered if it is exposed to temperatures of 0° or below. Freeman (1966) stated that T. paludosa larvae have surprisingly little cold-hardiness, after he had found mortalities of 8.3% after 60 h at -2.5° and 61.5% after 10 h at -5°. The leatherjacket's susceptibility to invasion by TIV is possibly increased after exposure to such low temperatures, possibly by the formation in the gut of small ice crystals which puncture the peritrophic membrane, allowing the virus access to the underlying cells.

Table 47. Mean monthly air temperatures at Shrewsbury (Shropshire) from September, 1953 to May, 1954.

TIV outbreak observed in 1954 (up to 15%).

Month	Mean air temperature (°C)	Deviation from 1931-60 mean (°C)
1953 Sept.	14.0	+ 0.3
Oct.	9.1	- 0.9
Nov.	8.8	+ 2.0
Dec.	7.2	+ 2.4
1954 Jan.	3.1	- 0.4
Feb.	2.8	- 1.2
Mar.	6.3	+ 0.3
Apr.	7.8	- 0.8
May	11.1	- 0.4

Table 48. Mean monthly air temperatures at Stirling from September, 1962 to May, 1963. TIV outbreak observed in April, 1963 (75%).

Month	Mean air temperature (°C)	Deviation from 1931-60 mean (°C)
1962 Sept.	10.9	- 1.8
Oct.	9.6	+ 0.2
Nov.	4.7	- 1.3
Dec.	3.1	- 1.2
1963 Jan.	0.1	- 2.8
Feb.	0.1	- 3.6
Mar.	5.5	- 0.1
Apr.	7.8	- 0.3
May	10.0	- 0.9

Table 49. Mean monthly air temperatures at Stirling from September, 1965 to May, 1966. TIV outbreak observed in March, 1966 (20%).

Month	Mean air temperature (°C)	Deviation from 1931-60 mean (°C)
1965 Sept.	12.1	- 0.6
Oct.	9.7	+ 0.3
Nov.	3.8	- 2.2
Dec.	2.9	- 1.4
1966 Jan.	2.7	- 0.2
Feb.	3.4	- 0.3
Mar.	7.1	+ 1.5
Apr.	5.6	- 2.5
May	10.9	0.0

It must be stressed that these considerations are highly speculative, and it could equally well be argued that the TIV outbreak in Shropshire in 1954 was due to the extremely mild conditions prevailing in the November and December of the previous year (Table 47).

The second possible factor determining the incidence of TIV epizootics is population density. Xeros (1956) stated that the TIV outbreak which he observed was in a low density population of leatherjackets, whereas both the outbreaks observed by Newbold, J.W. (personal communication) were in high density populations, there being 4.9 million and 2.5 - 3.7 million leatherjackets per hectare in the 1963 and 1966 outbreaks respectively.

It would be expected that higher population densities would enhance the transmission of the virus as there is an increased chance of moribund TIV-infected larvae being discovered and ingested by the healthy larvae. In Experiment 8 there was no evidence that higher population densities contributed to a greater proportion of larvae becoming infected, but the larvae were not confined together until the fourth instar. It is probable that a population consisting of infected and non-infected larvae from the first-instar might well undergo an increase in the proportion of infected larvae in later instars. Stairs (1965c) concluded that NPV epizootics of the forest tent caterpillar, Malacosoma disstria (Hübner), may develop naturally from an extremely small amount of virus provided that this reaches the population while the larvae are still in an early instar.

It is commonly found in ecosystems that if an organism increases in number, then the parasites and predators of that organism also increase in number and that this increase often lags behind that of the host. For

example, Utida (1957) demonstrated that numbers of parasitic wasps often lag behind numbers of bean weevils. There may be a steady rise in the numbers of the host, and in tipulids this may take several years. The increase in the numbers of parasites and predators may eventually result in a decrease of the host population.

A gradual increase in the population size is possibly a prelude to a TIV epizootic. In the 1963 epizootic in Stirlingshire, 75% of a population of 4.9 million larvae per hectare were found to be infected with TIV in April. By May the population had crashed to 124,000 larvae per hectare, with no evidence of TIV infection in the survivors. This represents a drop in numbers of 97.5%, which suggests that, although TIV was the main cause of the population crash, there were probably additional factors operating. It is probable that other parasites and also predators of the leatherjacket underwent an increase in numbers along with TIV. Stewart (1969) found that both insect and microbial parasites of tipulids were most common in dense populations, although many dense populations were free from parasites.

The resistance of the host to infection is the third possible factor which may affect the incidence of TIV epizootics. Martignoni and Schmid (1961) discussed the resistance of insects to viruses in connection with the population density: 'A virus epizootic reduces a population to a lower density, while the population resistance to the virus disease increases, and its heterogeneity in this respect decreases'. Populations in phases of increasing density are found to be less resistant and more heterogeneous in their response to parasites. As the heterogeneity of the population gradually increases, so the resistance of the population decreases.

Results obtained during the present work, for example in Experiment 2, suggest that individual leatherjackets display a wide variation in their resistance to infection with TIV. Stairs (1965a) found similar variation in the resistance of Galleria mellonella larvae to infection with NPV. Bucher (1956) stated that such variation tends to limit the usefulness of microbes for biological control, but insects may display similar variation in resistance to chemical insecticides, thus reducing the effectiveness of these substances.

A factor which possibly limits the development of a TIV epizootic is predation by birds. Newbold, J.W. (personal communication) observed birds feeding on the TIV-infected larvae which had come to the surface in the 1963 epizootic in Stirlingshire. Birds may thus remove a source of infection for the healthy larvae.

In this instance the leatherjacket population was reduced from a level which is highly damaging to the crop, to one which causes insignificant damage. At least 75% of this total reduction of 97.5% was due to the TIV epizootic. A reduction of this order compares favourably with reductions of 95.1% and 98.4% achieved by Willis (1963) in trials with DDT and aldrin respectively.

Of the 3 factors discussed, namely temperature, population resistance and population density, only the latter can be influenced by man. An increase in population density caused by the introduction of TIV-infected larvae may encourage an epizootic. Microbial control measures are concerned with artificially induced epizootics and/or acceleration in the rate of development of natural epizootics by shortening the time lag between the increase in numbers of the host and that of its parasite.

Induction of TIV epizootics

a) Methods for applying the virus

There are 4 possible methods whereby the level of TIV could be increased in the leatherjacket's environment: by spraying with a suspension of the virus; by scattering virus-impregnated bait; by releasing live infected leatherjackets; and by scattering dead infected leatherjackets. Most insect viruses which have been used for biological control have been applied to the crop which required protection either as a dust or a spray, an adjuvant often being added to sprays in order to cause the virus to adhere more strongly to the vegetation (see Ignoffo, 1968).

Bucher (1956) and Smith and Rivers (1956) have stated that soil-dwelling insects, such as leatherjackets, are more difficult to control than those which live and feed on the aerial portions of plants. It has been established, however, that leatherjackets emerge from the soil at night to feed on the leaves and stems of plants (Cameron, 1945), which may be pulled back into the soil (Willis, 1963), so that virus on the leaf surfaces could be ingested. It is possible, however, that doses of virus received in this way are insufficient to cause infection (see Experiments 13 and 14). Leatherjackets could also ingest the virus by drinking from water droplets containing it. Few fourth-instar larvae are likely to become infected in this way (Experiments 13 and 14), but first-instar larvae may possibly be more susceptible.

Bran baits impregnated with insecticides are used in the control of leatherjackets to increase the probability of the insects ingesting a lethal dose of the chemical, and they have been shown to be slightly more efficient than sprays (White, 1967). Leatherjackets might ingest lethal

doses of the virus if they fed on TIV-impregnated baits. Before testing for this possibility it would be necessary to determine the effect on the infectivity of the virus of desiccation, either before or after mixing with the bait.

It has been demonstrated that a leatherjacket can receive an infective dose of TIV by feeding on an infected cadaver (Experiment 11), and it may be that the best way to apply the virus in the field is to disseminate infected insects, either alive or dead. The release of live virus-infected individuals to disperse insect viruses among populations of the host was suggested by Martignoni and Milstead (1962). Gilmore and Tashiro (1966) successfully disseminated the noninclusion virus of the citrus red mite, Panonychus citri (McGregor), among field populations by releasing live infected mites.

If live TIV-infected leatherjackets are to be released in the field, then it is necessary that they should be in a well-advanced stage of the disease, otherwise they might cause additional damage to the crop. It was observed that most TIV-infected T. oleracea larvae continued to feed until they were almost moribund. Ahmed (1968) made similar observations with TIV-infected T. paludosa larvae. For biological control purposes it would be necessary to ensure that the diseased larvae were all approaching the moribund state at the time they were required. On the other hand, if larvae which had died from TIV infection were employed, they could be prepared in advance and stored until required.

It is probable that TIV remains infective for long periods in infected larval cadavers. Cadavers which had been stored in the refrigerator for

several weeks were still highly infective (Experiment 11), and Glitz *et al* (1968) observed no alteration in the physical integrity of TIV particles after extensive incubation with the enzymes pronase, trypsin, papain and pancreatic deoxyribonuclease. One problem that might be encountered with the dissemination of either live or dead TIV-infected larvae is that many may be ingested by birds. Many living larvae in an advanced stage of the disease might remain on the surface rather than burrow into the soil.

b) Timing of virus applications

The timing of any TIV application to the field would probably be critical. It would be necessary to attempt to establish the disease in the insect population as soon as possible after the larvae had hatched, before they became large enough to damage the plants significantly, and before the temperature dropped to a level where virus replication is greatly retarded (Experiments 21 and 22).

TIV does not kill its host rapidly. The LD50 has been given as 14-28 days by Rivers and Glitz (personal communication to Ignoffo, 1968), but in fact it is highly variable, depending upon the virus dose (Experiment 2), inoculation route (Experiment 13) and temperature (Experiments 21 and 22). Survival times were generally longer when infection occurred *per os* and at lower temperatures. In fact, a short survival time of infected larvae would reduce the possibility of transmission of the virus to the subsequent generation and, therefore, the relatively long period between infection and death has survival value for the virus. If control measures were not commenced until damage began to appear, further damage would be sustained before the insects were eventually killed by the virus.

The optimum timing depends upon the tipulid species for which control is desired. T. paludosa, the most common pest species, oviposits in late summer (Ahmed, 1968), so that the best time for virus application might be in the early autumn. T. oleracea on the other hand is bivoltine, ovipositing in May and August (Freeman, 1967), so that it would probably be necessary to begin control measures earlier in this case.

Stewart (1969) concluded that additional tipulid species, such as Tipula vernalis (Meigen) and Nephrotoma flavescens (Linnaeus) may also cause agricultural damage. It would therefore be necessary to identify which tipulid species were present, and to study their life-cycles before attempting to control them. Forecasts of probable leatherjacket damage would be essential for biological control as the virus should be applied before any visible damage occurs. Such forecasts are already made for chemical control measures.

Mass production of TIV

If TIV is found to be a suitable agent for biological control of leatherjackets, then it will be necessary to produce the virus on a large scale. The cost must compare fairly favourably with that for chemical control, which is approximately £1.85 per hectare (Long, 1969).

If the virus were to be used in a spray or a bait, there are many insect species in which it could be propagated; T. oleracea and Pieris brassicae are two susceptible species which are easily reared in large numbers. Further advances in techniques of insect tissue culture may render feasible the large-scale production of TIV in cell cultures. If the dissemination of live or dead TIV-infected leatherjackets is found to be the best way of initiating TIV epizootics, then it will be necessary to produce large numbers of TIV-infected tipulid larvae.

Tests for undesirable effects of TIV

Before any infective agent is released into the environment for purposes of biological control, it is important that it should first be exhaustively tested for any harmful effects against vertebrates, including man, beneficial invertebrates and plants. In the United States of America a microbial control agent must be approved as safe by the Pesticide Regulation Division, U.S. Department of Agriculture (Heimpel, 1967).

Although some viruses are highly specific, there are many which infect a wide range of hosts, including the arboviruses, which infect both a vertebrate and an invertebrate host, and some plant viruses which infect both a plant and an invertebrate host. It is therefore important to test any microbe against a wide range of animals and plants before disseminating it in the environment. Even an agent which is shown to have no harmful effects should be kept under observation for the appearance of mutants with infectivity or toxicity towards organisms other than the insect pest.

A number of insect viruses have been tested for harmful effects in vertebrates. Ignoffo and Heimpel (1965) collected data from scientists working on the serology of insect viruses: 28 viruses had been injected into 6 vertebrate species without a single animal exhibiting toxic reactions. Heimpel (1967) pointed out that most viruses ingested by vertebrates are rapidly inactivated in the hostile acid environment of the stomach. Heimpel and Buchanan (1967) fed Heliothis zea NPV to 10 men and women, who were each subsequently given a thorough medical examination: no significant changes were found. Barnes et al (1970) tested the same virus for any possible carcinogenic effects in rats, but none were found.

Steinhaus (1951), Aizawa (1961) and Cantwell, Faust and Poole (1968)

inoculated insect viruses into chick embryos, but found no evidence of viral replication. In testing for harmful effects towards beneficial insects Knox (1970) fed 2 granulosis viruses and 7 NPVs to honeybees; he found no difference between treated and untreated colonies.

There have been a few reports of the replication in vertebrate cells of viruses which normally infect only insects, but these have yet to be confirmed. Smirnova (1966) and Alekseenko and Kolomiets (quoted by Cantwell et al, 1968) claimed that they grew the sacbrood virus and a paralysis virus of bees, respectively, in chick embryos, and Himeno et al (1967) described the formation of polyhedra containing infective virus in FL cells (derived from the human amnion) infected with DNA from silkworm NPV.

TIV has been tested against a number of organisms. Antibodies against TIV have been produced in rabbits on a number of occasions, e.g. Cunningham and Tinsley (1968), Glitz et al (1968) and in the present investigations, without any reports of adverse effects in the animals. Attempts to transmit TIV to crayfish (Rivers, C.F., unpublished, quoted by Ignoffo and Heimpel, 1965) and tadpoles (Rivers, C.F., personal communication) were unsuccessful, and Stobbart, R.H. (personal communication) was unable to infect HeLa cells with TIV.

If TIV is to be used for biological control it will be necessary to carry out further tests on a range of organisms, and in particular on beneficial insects, including the insect predators and parasites of tipulids, in view of the wide experimental host range of the virus and the report by Malyuta and Aleksandrov (1969) of the induction of lethal mutations in Drosophila melanogaster by TIV.

Resistance of the insect to TIV

The T. oleracea strain studied in Experiment 22a had an altered response to TIV in that the time taken to kill the infected insect was longer than normal. A greater problem while using the virus for biological control would be the appearance of insect strains with increased resistance to the virus, and in fact it must be expected that this will occur.

The experimental results suggest that T. oleracea larvae display wide variation in their resistance to TIV, so that when the insects are exposed to the virus the more resistant strains will tend to be selected. Stairs (1965a) suggested that such variation may be beneficial to both virus and host. At no time is the host population completely resistant, thus eliminating the virus, or completely susceptible, eliminating both. The selection of resistant insects also presents a problem in the use of chemical insecticides. If such a problem is encountered during a programme of biological control it may be possible to overcome it by isolating a strain of the pathogen with increased virulence towards the more resistant insect strain, or by the introduction of an alternative parasite.

Conclusion

The initiation of TIV epizootics may present difficulties, in view of the large dose of TIV which leatherjackets must ingest in order to become infected. It may be that one of the other parasites or a predator of leatherjackets is more suitable than TIV as an agent of biological control. Alternatively, control may best be achieved with a combination of two or more of the parasites and/or predators. The NPV of tipulids merits inclusion in further investigations since, although the infected

leatherjacket is not killed rapidly, it stops feeding shortly after becoming infected (Ahmed, 1968), in contrast to the TIV-infected leatherjacket.

The experiments described in this report have indicated how TIV may be transmitted in nature and how seasonal variations in temperature may affect the course of the disease. In order to determine whether the potential of TIV as an agent for the biological control of leatherjackets has practical value these experiments must now be followed by further experiments out-of-doors, and then by field trials.

APPENDIX

1. St. Ives fluid

160 ml orthodichlorobenzene

40 ml 10 per cent sodium oleate in water

50 ml Jeyes fluid

15 ml oleic acid

Stirred, then 6 ml NaOH added.

Cresol added until a homogeneous solution is obtained.

Diluted 1/400 with water before use.

2. Salts and vitamins solution

Salt mixture (Beckman, Bruckart and Reiser, 1953).

$CaCO_3$	120 g
K_2HPO_4	129 g
$CaHPO_4.2H_2O$	30 g
$MgSO_4.7H_2O$	40.8 g
NaCl	68 g
$FeC_6H_5O_7.5H_2O$	11 g
KI	0.32 g
$MnSO_4.4H_2O$	2.0 g
$ZnCl_2$	0.10 g
$CuSO_4.5H_2O$	0.12 g

Vitamin stock

nicotinic acid	600 mg
calcium pantothenate	600 mg
riboflavin	300 mg
aneurine hydrochloride	150 mg
pyridoxine hydrochloride	150 mg
folic acid	150 mg
D-biotin	12 mg
cyanocobalamin	1.2 mg

in 100 ml water.

Preparation of the solution

25 g salt mixture plus 5 l distilled water.

Autoclaved at 121° for 15 min; cooled.

10 ml vitamin stock added; filtered.

3. Insect saline with antibiotics (ISA)

Insect saline

NaCl	6.5 g
KCl	1.4 g
$CaCl_2$	0.12g
$NaHCO_3$	0.1 g
Na_2HPO_4	0.01g

Dissolved in 1 l distilled water.

Autoclaved at 121° for 15 min.

Penicillin stock solution

Twenty tablets (10,000 i.u./tablet) dissolved in 10 ml distilled water. Stored at -20°.

Aureomycin stock solution

0.1 g dissolved in 10 ml distilled water.

Stored at -20°.

Preparation of ISA

98 ml sterile insect saline

1 ml penicillin stock solution (final concentration 200 i.u./ml)

1 ml aureomycin stock solution (final concentration 0.1 mg/ml).

ACKNOWLEDGEMENTS

I would like to thank the following: the Principal and Governors of The West of Scotland Agricultural College for research facilities; colleagues in the College for useful discussions; the late Professor I.R.W. Lominski for supervision in the early stages of the work; Dr. S. Baines and Professor A.C. Wardlaw for helpful criticism of the manuscript; Mr. G.K. Shukla for assistance with statistical analyses; Dr. J.H. Freer for providing the electron micrograph; Miss Linda McCroskie and Miss Noreen McWhinnie for technical assistance at different stages of the work; Miss Linda Wood for competent typing of the thesis; and my wife for her interest, encouragement and inspiration throughout.

REFERENCES

Abu Salih, H.S., Murant, A.F. and Daft, M.J. (1968). The use of antibody-sensitised latex particles to detect plant viruses. Journal of General Virology, 3, 299-302.

Ahmed, M. (1968). A study on the Tipulidae (Diptera) of agricultural importance in south-east Scotland. Ph.D. thesis, University of Edinburgh.

Aizawa, K. (1953). Multiplication mode of the silkworm jaundice virus. I. On the multiplication mode in connection with the latent period and the LD50-time curve. Sanshi Shikensho Hokoku, 14, 201-228. (English summary).

Aizawa, K. (1959). Mode of multiplication of silkworm nuclear polyhedrosis virus. II. Multiplication of the virus in the early period of the LD50-time curve. Journal of Insect Pathology, 1, 67-74.

Aizawa, K. (1961). Change in the shape of silkworm polyhedra by means of passage through chick embryo. Entomophaga, 6, 197-201.

Aizawa, K. (1962). Antiviral substance in the gut-juice of the silkworm, Bombyx mori (Linnaeus). Journal of Insect Pathology, 4, 72-76.

Allison, A.C. and Burke, D.C. (1962). The nucleic acid contents of viruses. Journal of General Microbiology, 27, 181-194.

Anderson, J.F. (1970). An iridescent virus infecting the mosquito Aedes stimulans. Journal of Invertebrate Pathology, 15, 219-224.

Anderson, E.S., Armstrong, J.A. and Niven, J.S.F. (1959). Fluorescence microscopy: observation of virus growth with aminoacridines. 9th Symposium of the Society for General Microbiology, 224-255.

Anonymous (1969a). Strong measures in Sweden. Nature, 222, 3.

Anonymous (1969b). Pesticide residues in everyday foods. New Scientist, 41, 555.

Annual Report of the Insect Pathology Unit, Oxford (1969). Epidemiological studies on virus diseases of Tipula paludosa.

A.R.C. (1970). Third Report of the Research Committee on Toxic Chemicals, Agricultural Research Council, London.

Armstrong, J.A. and Niven, J.S.F. (1957). Histochemical observations on cellular and virus nucleic acids. Nature, 180, 1335-1336.

Aruga, H. (1957). Mechanism of virus resistance in the silkworm, Bombyx mori II. On the relation between the nuclear polyhedrosis and the cytoplasmic polyhedrosis. Nippon Sanshigaku Zasshi, 26, 279-283.

Aruga, H. and Hukuhara, T. (1960). Induction of nuclear and cytoplasmic polyhedroses by feeding of some chemicals to the silkworm. Nippon Sanshigaku Zasshi, 29, 44-49.

Bailey, L. (1965a). Paralysis of the honey bee, Apis mellifera Linnaeus. Journal of Invertebrate Pathology, 7, 132-140.

Bailey, L. (1965b). Susceptibility of the honey bee, Apis mellifera Linnaeus, infested with Acarapis woodi (Rennie) to infection by airborne pathogens. Journal of Invertebrate Pathology, 7, 141-143.

Bailey, L. (1969). The multiplication and spread of sacbrood virus of bees (Apis mellifera). Annals of Applied Biology, 63, 483-491.

Bailey, L. and Gibbs, A.J. (1964). Acute infection of bees with paralysis virus. Journal of Insect Pathology, 6, 395-407.

Bailey, L., Gibbs, A.J. and Woods, R.D. (1970). A simple way of purifying several insect viruses. Journal of General Virology, 6, 175-177.

Barnes, H.F. (1937). Methods of investigating the bionomics of the common crane-fly, Tipula paludosa Meigen, together with some results. Annals of Applied Biology, 24, 356-368.

Barnes, H.F. (1941). Sampling for leatherjackets with orthodichlorobenzene emulsion. Annals of Applied Biology, 28, 23-28.

Barnes, R.W., Meinecke, C.F., McClane, W.C. and Rehnborg, C.S. (1970). Long-term feeding and other toxicity - pathogenicity studies on rats using a commercial preparation of the nuclear - polyhedrosis virus of Heliothis zea. Journal of Invertebrate Pathology, 16, 112-115.

Bauer, D.J. (1960). Some applications of a single-dilution method of titrating neurotropic viruses in zero mortality (Do) units. British Journal of Experimental Pathology, 41, 130-139.

Beard, R.L. and Walton, G.S. (1969). Kojic acid as an insecticidal mycotoxin. Journal of Invertebrate Pathology, 14, 53-59.

Beckman, H.F., Bruckart, S.M. and Reiser, R. (1953). Laboratory culture of the pink bollworm on chemically-defined media. Journal of Economic Entomology, 46, 627.

Bellett, A.J.D. (1965). The multiplication of Sericesthis iridescent virus in cell cultures from Antheraea eucalypti Scott. II. An in vitro assay for the virus. Virology, 26, 127-131.

Bellett, A.J.D. (1967). Preliminary classification of viruses based on quantitative comparisons of viral nucleic acids. Journal of Virology, 1, 245-259.

Bellett, A.J.D. (1968). The iridescent virus group. Advances in Virus Research, 13, 225-246.

Bellett, A.J.D. and Fenner, F. (1968). Studies of base-sequence homology among some cytoplasmic deoxyriboviruses of vertebrate and invertebrate animals. Journal of Virology, 2, 1374-1380.

Bellett, A.J.D. and Inman, R.B. (1967). Some properties of deoxyribonucleic acid preparations from Chilo, Sericesthis and Tipula iridescent viruses. Journal of Molecular Biology, 25, 425-432.

Bellett, A.J.D. and Mercer, E.H. (1964). The multiplication of Sericesthis iridescent virus in cell cultures from Antheraea eucalypti Scott. I. Qualitative experiments. Virology, 24, 645-653.

Bergold, G.H. (1951). The polyhedral disease of the spruce budworm, Choristoneura fumiferana (Clem.) (Lepidoptera : Tortricidae). Canadian Journal of Zoology, 29, 17-23.

Bergold, G.H. (1952). The multiplication of insect viruses. Symposium of the Society for General Microbiology, 2, 276-283.

Bergold, G.H. (1953). Insect viruses. Advances in Virus Research, 1, 91-139.

Bird, F.T. (1953). The use of a virus disease in the biological control of the European pine sawfly, Neodiprion sertifer (Geoffr.). Canadian Entomologist, 85, 437-446.

Bird, F.T. (1955). Virus diseases of sawflies. Canadian Entomologist, 87, 124-127.

Bird, F.T. (1961a). The development of Tipula iridescent virus in the crane fly, Tipula paludosa Meig., and the wax moth, Galleria mellonella L. Canadian Journal of Microbiology, 7, 827-830.

Bird, F.T. (1961b). Transmission of some insect viruses with particular reference to ovarial transmission and its importance in the development of epizootics. Journal of Insect Pathology, 3, 352-380.

Bird, F.T. (1962). On the development of the Tipula iridescent virus particle. Canadian Journal of Microbiology, 8, 533-534.

Bird, F.T. and Burk, J.M. (1961). Artificially disseminated virus as a factor controlling the European spruce sawfly, Diprion hercyniae (Htg.), in the absence of introduced parasites. Canadian Entomologist, 93, 228-238.

Black, L.M. (1949). Multiplication of clover club-leaf virus (Aureogenus clavifolium) in its insect vector. Phytopathology, 39, 2-3.

Bonhoeffer, F. and Schachman, H.K. (1960). Studies on the organization of nucleic acids within nucleoproteins. Biochemical and Biophysical Research Communications, 2, 366-371.

Bregliano, J.-C. (1969). Relations du virus sigma avec son hote: Contribution à l'étude du caractère g^-. (Relationships of the sigma virus with its host: Contribution to the study of the g^- character strain). Annales de l'Institut Pasteur, 117, 335-338. (Biological Abstracts (1970), 51, no. 58193).

British Agrochemicals Association (1970), in 'First Review of Insecticide and Fungicide Usage,' ed. Stell, G. British Crop Protection Council, Monograph No. 1.

Brown, E.S. and Swaine, G. (1965). Virus disease of the African armyworm, Spodoptera exempta (Wlk.). Bulletin of Entomological Research, 56, 95-116.

Bucher, G.E. (1956). General summary and review of utilisation of disease to control insects. Proceedings of the Tenth International Congress of Entomology, Montreal, 4, 695-701.

Burgdorfer, W. and Varma, M.G.R. (1967). Trans-stadial and transovarial development of disease agents in arthropods. Annual Review of Entomology, 12, 347-376.

Burton, J. (1968). 'The Oxford Book of Insects', Oxford University Press.

Cameron, A.E. (1945). Insect pests of 1944. Transactions of the Highland and Agricultural Society of Scotland, 57, 54-72.

Canerday, T.D. and Arant, F.S. (1968). Pathogen - host - environment relationships in a cabbage-looper nuclear polyhedrosis. Journal of Invertebrate Pathology, 12, 344-348.

Cantwell, G.E., Faust, R.M. and Poole, H.K. (1968). Attempts to cultivate insect viruses in avian eggs. Journal of Invertebrate Pathology, 10, 161-162.

Carson, R. (1962). 'Silent Spring', Houghton Mifflin.

Chamberlain, R.W. (1968). Arboviruses, the arthropod - borne animal viruses. in 'Insect Viruses', ed. Maramorosch, K., Current Topics in Microbiology and Immunology, 42, 38-58.

Chamberlain, R.W. and Sudia, W.D. (1961). Mechanism of transmission of viruses by mosquitoes. Annual Review of Entomology, 6, 371-390.

Chapman, H.C., Clark, T.B., Woodard, D.B. and Kellen, W.R. (1966). Additional mosquito hosts of the mosquito iridescent virus. Journal of Invertebrate Pathology, 8, 545-546.

Clark, E.C. (1958). Ecology of the polyhedroses of tent caterpillars. Ecology, 39, 132-139.

Clark, T.B., Kellen, W.R. and Lum, P.T.M. (1965). A mosquito iridescent virus (MIV) from Aedes taeniorhynchus (Wiedemann). Journal of Invertebrate Pathology, 7, 519-521.

Cliver, D.O. (1968). Virus interactions with membrane filters. Biotechnology and Bioengineering, 10, 877-889.

Conte, A. (1907). Compte Rendu de l'Association Française pour l'Avancement des Sciences, 36, 622-623.

Counce, S.J. (1961). The analysis of insect embryogenesis. Annual Review of Entomology, 6, 295-312.

Coulson, J.C. (1962). The biology of Tipula subnodicornis Zetterstedt, with comparative observations on Tipula paludosa Meigen. Journal of Animal Ecology, 31, 1-21.

Cunningham, J.C. and Tinsley, T.W. (1968). A serological comparison of some iridescent non-occluded insect viruses. Journal of General Virology, 3, 1-8.

Curtis, J. (1849). Observations on the natural history and economy of various insects affecting the potato-crops, including plant-lice, plant-bugs, frog-flies, caterpillars, crane-flies, wireworms, millipedes, mites, beetles, flies, etc. Journal of the Royal Agricultural Society, 10, 70-118.

David, W.A.L. and Gardiner, B.O.C. (1965a). Rearing Pieris brassicae L. larvae on a semi-synthetic diet. Nature, 207, 882-883.

David, W.A.L. and Gardiner, B.O.C. (1965b). Resistance of Pieris brassicae (Linnaeus) to granulosis virus and the virulence of the virus from different host races. Journal of Invertebrate Pathology, 7, 285-290.

Dawson, R.B. (1932). Leather Jackets. Journal of Greenkeeping Research, 2, 183-195.

Dawson, R.B. and Ferro, R.B. (1936). Investigations on the control of leatherjackets. (1) The use of lead arsenate. Journal of Greenkeeping Research, 4, 239-261.

Day, M.F. (1965). Sericesthis iridescent virus infection of the adult Galleria. Journal of Invertebrate Pathology, 7, 102-105.

Day, M.F. and Dudzinski, M.L. (1966). The effect of temperature on the development of Sericesthis iridescent virus. Australian Journal of Biological Science, 19, 481-493.

Day, M.F. and Gilbert, N. (1967). The number of particles of Sericesthis iridescent virus required to produce infections of Galleria larvae. Australian Journal of Biological Science, 20, 691-693.

Day, M.F. and Mercer, E.H. (1964). Properties of an iridescent virus from the beetle, Sericesthis pruinosa. Australian Journal of Biological Science, 17, 892-902.

Day, M.F. and Powning, R.F. (1949). A study of the processes of digestion in certain insects. Australian Journal of Scientific Research, Series B, 2, 175-215.

Doane, C.C. (1969). Trans-ovum transmission of a nuclear-polyhedrosis virus in the gypsy moth and the inducement of virus susceptibility. Journal of Invertebrate Pathology, 14, 199-210.

Dougherty, R.M., McCloskey, R.V. and Stewart, R.B. (1960). Analysis of the single dilution method of titration of psittacosis virus. Journal of Bacteriology, 79, 899-903.

Drake, E.L. and McEwen, F.L. (1959). Pathology of a nuclear polyhedrosis of the cabbage looper, Trichoplusia ni (Hübner). Journal of Insect Pathology, 1, 281-293.

Dunn, E. (1966). The leatherjacket problem in south-east Scotland. Conference of research workers on leatherjackets, ARC 704/66.

Dutky, S.R. (1959). Insect microbiology. Advances in Applied Microbiology, 1, 175-200.

Edwards, C.A. and Heath, G.W. (1964). 'The Principles of Agricultural Entomology.' Chapman and Hall, London.

Escritt, J.R. (1947). Investigations on the control of leatherjackets. 4. Trials with DDT and gammexane. Journal of the Board of Greenkeeping Research, 7, 80-90.

Finney, D.J. (1952). 'Probit Analysis', second edition. Cambridge University Press.

Freeman, B.E. (1964). A population study of Tipula species (Diptera, Tipulidae). Journal of Animal Ecology, 33, 129-140.

Freeman, B.E. (1966). Cold hardiness and resistance to desiccation in Tipuline larvae. Conference of Research Workers on Leatherjackets. ARC 735/66.

Freeman, B.E. (1967). Studies on the ecology of larval Tipulinae (Diptera, Tipulidae). Journal of Animal Ecology, 36, 123-146.

Fukaya, M. and Nasu, S. (1966). A Chilo iridescent virus from Chilo suppressalis Walker (Lepidoptera : Pyralidae). Japanese Journal of Applied Entomology and Zoology, 1, 69-72.

Fytizas, E. (1968). The effect of actidione (cycloheximide) on the adults of Dacus oleae Gmel (Diptera : Tephritidae). Annales des épiphyties, 19, 261-265. (Biological Abstracts (1969), 50, no. 78650).

Gard, S. (1940). Encephalomyelitis of mice. II. A method for the measurement of virus activity. Journal of Experimental Medicine, 72, 69-77.

Gibbs, A.J. (1968). Insect entero-viruses. Virus Group Meeting, Society for General Microbiology, London.

Gibbs, A.J., Harrison, B.D., Watson, D.H. and Wildy, P. (1966). What's in a virus name?. Nature, 209, 450-454.

Gilmore, J.E. and Tashiro, H. (1966). Fecundity, longevity, and transinfectivity of citrus red mites (Panonychus citri) infected with a noninclusion virus. Journal of Invertebrate Pathology, 8, 334-339.

Glitz, D.G., Hills, G.J. and Rivers, C.F. (1968). A comparison of the Tipula and Sericesthis iridescent viruses. Journal of General Virology, 3, 209-220.

Gravell, M. and Granoff, A. (1970). Viruses and renal carcinoma of Rana pipiens. IX. The influence of temperature and host cell on replication of frog polyhedral cytoplasmic deoxyribovirus (PCDV). Virology, 41, 596-602.

Grennan, E.J. (1966). Pasture damage by leatherjacket grubs. Irish Journal of Agricultural Research, 5, 145-146.

Hale, L.J. (1965). 'Biological laboratory data', 2nd ed. Methuen, London.

Hall, I.M. (1957). Use of a polyhedrosis virus to control cabbage looper on lettuce in California. Journal of Economic Entomology, 50, 551-553.

Harpaz, I. and Ben Shaked, Y. (1964). Generation-to-generation transmission of the nuclear-polyhedrosis virus of Prodenia litura (Fabricius). Journal of Insect Pathology, 6, 127-130.

Harrap, K.A. and Robertson, J.S. (1968). A possible infection pathway in the development of a nuclear polyhedrosis virus. Journal of General Virology, 3, 221-5.

Harvey, T.L. and Howell, D.E. (1965). Resistance of the house fly to Bacillus thuringiensis Berliner. Journal of Invertebrate Pathology, 7, 92-100.

Hayashiya, K., Nishida, J. and Matsubara, F. (1969). Inactivation of nuclear polyhedrosis virus in the digestive juice of silkworm larvae, Bombyx mori L.: I. Comparison of anti-viral activities in the digestive juices of larvae reared on natural and artificial diets. Japanese Journal of Applied Entomology and Zoology, 12, 189-193. (Biological Abstracts (1970), 51, no. 34936).

Heimpel, A.M. (1955). The pH in the gut and blood of the larch sawfly, Pristophora erichsonii (Htg.), and other insects with reference to the pathogenicity of Bacillus cereus Fr. and Fr. Canadian Journal of Zoology, 33, 99-106.

Heimpel, A.M. (1967). Progress in developing insect viruses as microbial control agents. Proceedings of the Joint United States-Japan Seminar on Microbial Control of Insect Pests, Fukuoka, pp. 51-61.

Heimpel, A.M. and Buchanan, L.K. (1967). Human feeding tests using a nuclear-polyhedrosis virus of Heliothis zea. Journal of Invertebrate Pathology, 9, 55-57.

Henry, J.E. and Jutila, J.W. (1966). The isolation of a polyhedrosis virus from a grasshopper. Journal of Invertebrate Pathology, 8, 417-418.

Hills, G.J. and Smith, K.M. (1959). Further studies on the isolation and crystallisation of insect cytoplasmic viruses. Journal of Insect Pathology, 1, 121-128.

Himeno, M., Sakai, F., Onodera, K., Nakai, H., Fukada, T. and Kawade, Y. (1967). Formation of nuclear polyhedral bodies and nuclear polyhedrosis virus of silkworm in mammalian cells infected with viral DNA. Virology, 33, 507-512.

Hostetter, D.L. and Biever, K.D. (1970). The recovery of virulent nuclear-polyhedrosis virus of the cabbage looper, Trichoplusia ni, from the feces of birds. Journal of Invertebrate Pathology, 15, 173-176.

Huger, A. (1959). Histological observations on the development of crystalline inclusions of the rickettsial disease of Tipula paludosa Meigen. Journal of Insect Pathology, 1, 60-66.

Huger, A. (1961). Licht- und Elektronenmikroskopische Untersuchungen über eine neue Haplosporidiase von Tipula paludosa Meigen. Proceedings of the First International Conference on Protozoology, Prague, 1961. pp. 506-509.

Hukuhara, T. (1962). Generation-to-generation transmission of the cytoplasmic polyhedrosis virus of the silkworm, Bombyx mori (Linnaeus). Journal of Insect Pathology, 4, 132-135.

Hukuhara, T. (1964). Induction of epidermal tumour in Bombyx mori (Linnaeus) with Tipula iridescent virus. Journal of Insect Pathology, 6, 246-248.

Hukuhara, T. and Aruga, H. (1959). Induction of polyhedrosis by temperature treatment in the silkworm (Bombyx mori L.). Nippon Sanshigaku Zasshi, 28, 235-241. (English summary.)

Hukuhara, T. and Hashimoto, Y. (1966a). Aggregation of Tipula iridescent virus with polystyrene latex particles. Journal of Invertebrate Pathology, 8, 540-542.

Hukuhara, T. and Hashimoto, Y. (1966b). Development of Tipula iridescent virus (TIV) in the silkworm, Bombyx mori L. (Lepidoptera : Bombycidae). Japanese Journal of Applied Entomology and Zoology, 1, 166-172. (Biological Abstracts (1970), 51, no. 5757.).

Hukuhara, T. and Hashimoto, Y. (1967). Multiplication of Tipula and Chilo iridescent viruses in cells of Antheraea eucalypti. Journal of Invertebrate Pathology, 9, 278-281.

Hurpin, B. (1968). The influence of temperature and larval stage on certain diseases of Melolontha melolontha. Journal of Invertebrate Pathology, 10, 252-262.

Ignoffo, C.M. (1964). Bioassay technique and pathogenicity of a nuclear-polyhedrosis virus of the cabbage looper, Trichoplusia ni (Hübner). Journal of Insect Pathology, 6, 237-245.

Ignoffo, C.M. (1965). The nuclear polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricius). IV. Bioassay of virus activity. Journal of Invertebrate Pathology, 7, 315-319.

Ignoffo, C.M. (1966). Effects of temperature on mortality of Heliothis zea larvae exposed to sublethal doses of a nuclear-polyhedrosis virus. Journal of Invertebrate Pathology, 8, 290-292.

Ignoffo, C.M. (1968). Viruses - living insecticides. in 'Insect Viruses', ed. Maramorosch, K., Current Topics in Microbiology and Immunology, 42, 129-167.

Ignoffo, C.M. and Heimpel, A.M. (1965). The nuclear-polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricius). V. Toxicity - pathogenicity of virus to white mice and guinea pigs. Journal of Invertebrate Pathology, 7, 329-340.

Jaques, R.P. (1962). The transmission of nuclear-polyhedrosis virus in laboratory populations of Trichoplusia ni (Hübner). Journal of Insect Pathology, 4, 433-445.

Jaques, R.P. (1967). The persistence of a nuclear polyhedrosis virus in the habitat of the host insect, Trichoplusia ni : II. Polyhedra in soil. Canadian Entomologist, 99, 820-829.

Jensen, S., Johnels, A.G., Olsson, M. and Otterlind, G. (1969). DDT and PCB in marine animals from Swedish waters. Nature, 224, 247-250.

Kalmakoff, J. and Tremaine, J.H. (1968). Physicochemical properties of Tipula iridescent virus. Journal of Virology, 2, 738-744.

Kanyuka, V. Yu. (1965). Aktyvatsiya latentnoho virusu yadernoho poliedrozu pid vplyvom raiduzhnoho virusu dovhonizhky. (Activation of the latent virus of nuclear polyhedrosis through the injection of Tipula iridescent virus). Mykrobiolohichniyi Zhurnal Akademia Nauk Ukrainska RSR, 27, 77-80. (Biological Abstracts (1967), 48, no. 65686.).

Kanyuka, V. Yu. (1968). Histokhimichne doslidzhennya komakh, zarazhenykh raiduzhnym virusom dovhonizhky. (Histochemical study of insects infected by iridescent virus of a crane-fly). Mykrobiolohichniyi Zhurnal (Kyyiv), 30, 348-354. (Biological Abstracts (1969), 50, no. 16797).

Kanyuka, V. Yu. and Pronina, A.D. (1970). Vyvchennya tsytopatolohichnykh zmin u tkanynakh komakh, zarazhenykh raiduzhnym virusom dovhonizhky (RVD). (Study of cytopathological changes in tissues of insects infected with Tipula iridescent virus.) Mykrobiolohichniyi Zhurnal (Kyyiv), 32, 263-268. (Biological Abstracts (1970), 51, no. 99076).

Kawase, S. and Hukuhara, T. (1967). Amino acid composition of the Tipula iridescent virus. Journal of Invertebrate Pathology, 9, 273-274.

Kelsey, J.M. (1958). Control of Pieris rapae by granulosis viruses. New Zealand Journal of Agricultural Research, 1, 778-782.

Kleczkowski, A. (1968). Methods of inactivation by ultraviolet radiation. in 'Methods in Virology', Volume 4, ed. Maramorosch, K. and Koprowski, H., pp. 93-138, Academic Press, New York and London.

Klug, A., Franklin, R.E. and Humphreys-Owen, S.P.F. (1959). The crystal structure of Tipula iridescent virus as determined by Bragg reflection of visible light. Biochimica et Biophysica Acta, 32, 203-219.

Knox, D.A. (1970). Tests of certain insect viruses on colonies of honeybees. Journal of Invertebrate Pathology, 16, 152.

Kobayashi, M., Yamaguchi, S. and Yokoyama, Y. (1969). Effect of the larval development on the susceptibility of the silkworm, Bombyx mori L. to the nuclear-polyhedrosis virus. Journal of Sericulture Science, Japan, 38, 481-487.

Koval', E.Z. and Savchenko, Ye. M. (1967). Tsikavi hifal'ni hryby na komarakh-dov-honohakh z Ukrayins' koyi RSR. (Interesting hyphal fungi on the crane-flies from the Ukranian SSR.) Dopovidi Akademiyi Nauk Ukrainskoyi RSR, 29, 842-844. (Biological Abstracts (1968), 49, no. 124561.)

Krieg, A. (1955). Untersuchungen über die Polyhedrose von Neodiprion sertifer (Geoffr.). Archiv für die gesamte Virusforschung, 6, 163-174.

Laigo, F.M. and Tamashiro, M. (1966). Virus and insect parasite interaction in the lawn-armyworm, Spodoptera mauritia acronyctoides (Guenee). Proceedings of the Hawaiian Entomological Society, 19, 233.

Laughlin, R. (1958a). The rearing of crane flies (Tipulidae). Entomologia experimentalis et applicata, 1, 241-245.

Laughlin, R. (1958b). Desiccation of eggs of the crane fly (Tipula oleracea L.). Nature, 182, 613.

Laughlin, R. (1960). Biology of Tipula oleracea L. : growth of the larva. Entomologia experimentalis et applicata, 3, 185-197.

Laughlin, R. (1967). Biology of T. paludosa : growth of the larva in the field. Entomologia experimentalis et applicata, 10, 52-68.

L'Héritier, Ph. (1958). The hereditary virus of Drosophila. Advances in Virus Research, 5, 195-245.

Linley, J.R. and Nielsen, H.T. (1968a). Transmission of a mosquito iridescent virus in Aedes taeniorhynchus. I. Laboratory experiments. Journal of Invertebrate Pathology, 12, 7-16.

Linley, J.R. and Nielsen, H.T. (1968b). Transmission of a mosquito iridescent virus in Aedes taeniorhynchus. II. Experiments related to transmission in nature. Journal of Invertebrate Pathology, 12, 17-24.

Long, E. (1969). The leatherjacket risk grows. Farmers' Weekly, Jan. 31st, 1969, (v) - (vi).

Lovibond, B. (1937). Investigations on the control of leatherjackets. (2) Notes on craneflies and their larvae. Journal of Greenkeeping Research, 5, 12-17.

MacDougall, R.S. (1932). Insect pests - No. XIII. Scottish Journal of Agriculture, 15, 48-58.

Malyuta, S.S. and Aleksandrov, Yu. M. (1969). Mutagenicity of some insect viruses. Cytol. Genet. (U.S.S.R.), 3, 266-269. (Virology Abstracts (1969), 3, V732).

Maramorosch, K. (1955a). Relative content of aster-yellows virus in inocula of vectors during four serial transfers. American Journal of Botany, 42, 676-678.

Maramorosch, K. (1955b). Multiplication of plant viruses in insect vectors. Advances in Virus Research, 3, 221-249.

Maramorosch, K. (1963). Arthropod transmission of plant viruses. Annual Review of Entomology, 8, 369-414.

Martignoni, M.E. (1957). Contributo alla conoscenza di una granulosi di Eucosma griseana (Hübner) (Tortricidae, Lepidoptera) quale fattore limitante il pullalamento dell'insetto nella Engadina alta. Mitteilungen der Schweizerischen Anstalt für das forstliche Versuchswesen, 32, 371-418.

Martignoni, M.E. (1964). Progressive nucleopolyhedrosis in adults of Peridroma saucia (Hübner). Journal of Insect Pathology, 6, 368-372.

Martignoni, M.E. and Milstead, J.E. (1962). Trans-ovum transmission of the nuclear polyhedrosis virus of Colias eurytheme Boisduval through contamination of the female genitalia. Journal of Insect Pathology, 4, 113-121.

Martignoni, M.E. and Schmid, P. (1961). Studies on the resistance to virus infections in natural populations of Lepidoptera. Journal of Insect Pathology, 3, 62-74.

Matta, J.F. (1970). The characterization of a mosquito iridescent virus. II. Physicochemical characterization. Journal of Invertebrate Pathology, 16, 157-164.

Matta, J.F. and Lowe, R.E. (1970). The characterization of a mosquito iridescent virus (MIV). I. Biological characteristics, infectivity, and pathology. Journal of Invertebrate Pathology, 16, 38-41.

Meats, A. (1967). The relation between soil water tension and rate of development of the eggs of Tipula oleracea and T. paludosa (Diptera, Nematocera). Entomologia experimentalis et applicata, 10, 394-400.

Meynell, G.G. and Meynell, E.W. (1958). The growth of micro-organisms in vivo with particular reference to the relation between dose and latent period. Journal of Hygiene, Cambridge, 56, 323-346.

Meynell, G.G. and Meynell, E.W. (1965). 'Theory and Practice in Experimental Bacteriology', Cambridge University Press.

Milne, A., Laughlin, R. and Coggins, R.E. (1965). The 1955 and 1959 population crashes in the leatherjacket, Tipula paludosa Meigen, in Northumberland. Journal of Animal Ecology, 34, 529-544.

Mitsuhashi, J. (1966). Appearance of iridescence in the tissues of the rice stem borer larvae, Chilo suppressalis Walker, infected with Chilo iridescent virus (Lepidoptera : Pyralidae). Japanese Journal of Applied Entomology and Zoology, 1, 130-137. (Biological Abstracts (1970), 51, no. 5755).

Mitsuhashi, J. (1967). Infection of leafhopper and its tissues cultivated in vitro with Chilo iridescent virus. Journal of Invertebrate Pathology, 9, 432-434.

Miyajima, S. and Kawase, S. (1967). Different infection response of silkworm larvae to the locality of cytoplasmic - polyhedrosis virus infection. Journal of Invertebrate Pathology, 9, 441-442.

Morris, O.N. (1962). Quantitative infectivity studies on the nuclear polyhedrosis of the western oak looper, Lambdina fiscellaria somniaria (Hulst). Journal of Insect Pathology, 4, 207-215.

Morris, O.N. (1967). A virus disease of Ectropis crepuscularia Schiff. (Geometridae : Lepidoptera). Canadian Journal of Microbiology, 13, 855-858.

Morris, O.N. (1970). Metabolic changes in diseased insects. III. Nucleic acid metabolism in Lepidoptera infected by densonucleosis and Tipula iridescent viruses. Journal of Invertebrate Pathology, 16, 180-186.

Müller - Kögler, E. (1958). Eine Rickettsiose von Tipula paludosa Meig. durch Rickettsiella tipulae, nov. spec. Naturwissenschaften, 45, 248-250.

Mulvany, J.G. (1969). Membrane filter techniques in microbiology. in "Methods in Microbiology", Vol. 1, Ch. 7. Ed. Norris, J.R. and Ribbons, D.W. Academic Press, London and New York.

Neilson, M.M. (1965). Effects of a cytoplasmic polyhedrosis on adult Lepidoptera. Journal of Invertebrate Pathology, 7, 306-314.

Neilson, M.M. and Elgee, D.E. (1960). The effect of storage on the virulence of a polyhedrosis virus. Journal of Insect Pathology, 2, 165-171.

Neilson, M.M. and Elgee, D.E. (1968). The method and role of vertical transmission of a nucleopolyhedrosis virus in the European spruce sawfly, Diprion hercyniae. Journal of Invertebrate Pathology, 12, 132-139.

Newsom, L.D. (1967). Consequences of insecticide use on nontarget organisms. Annual Review of Entomology, 12, 257-286.

Oliveira, A.R. and Ponsen, M.B. (1966). The development of a viral antigen in the hemocytes of Pieris brassicae inoculated with Tipula iridescent virus. Netherlands Journal of Plant Pathology, 72, 259-264.

Ossowski, L.L.J. (1957). The biological control of the wattle bagworm (Kotochalia junodi Heyl.) by a virus disease. I. Small-scale pilot experiments. Annals of Applied Biology, 45, 81-89.

Pimentel, D. and Shapiro, M. (1962). The influence of environment on a virus-host relationship. Journal of Insect Pathology, 4, 77-87.

Plus, N. (1954). Étude de la multiplication du virus de la sensibilité au gaz carbonique chez la Drosophile. Bulletin biologuique de la France et de la Belguique, 88, 248-293.

Prentice, I.W. and Woollcombe, T.M. (1951). Resolution of strawberry virus complexes. IV. The latent period of virus 3 in the vector. Annals of Applied Biology, 38, 389-394.

Putman, W.L. (1970). Occurrence and transmission of a virus disease of the European red mite, Panonychus ulmi. The Canadian Entomologist, 102, 305-321.

Rao, N.U. and Labzoffsky, N.A. (1969). A simple method for the detection of low concentration of viruses in large volumes of water by the membrane filter technique. Canadian Journal of Microbiology, 15, 399-403.

Reed, L.V. and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. American Journal of Hygiene, 27, 493-497.

Reeve, P. (1969). The study of virus virulence using Newcastle disease virus. Journal of General Microbiology, 59, xv - xvi.

Reinganum, C., O'Loughlin, G.T. and Hogan, T.W. (1970). A nonoccluded virus of the field crickets Teleogryllus oceanicus and T. commodus (Orthoptera : Gryllidae). Journal of Invertebrate Pathology, 16, 214-220.

Rennie, J. (1912). Note upon a tachinid parasite (Bucentes geniculata de Geer) of Tipula sp. Proceedings of the Royal Physical Society of Edinburgh, 18, 231.

Rennie, J. (1923). Polyhedral disease in Tipula paludosa Meig. Proceedings of the Royal Physical Society of Edinburgh, A20, 265-267.

Rennie, J. (1927). Crane fly grub and the oat crop. Scottish Journal of Agriculture, 10, 184-195.

Ricou, G. (1967). Recherches sur les populations de Tipules. Action de certains facteurs ecologiques sur Tipula paludosa Meig. Annales des épiphyties, 18, 451-481.

Rivers, C.F. (1966a). The virus diseases of Tipula paludosa Meigen. Conference of research workers on leatherjackets, ARC 732/66.

Rivers, C.F. (1966b). The natural and artificial dispersion of pathogens. in Proceedings of the International Colloquium on Insect Pathology and Biological Control, Wageningen, Netherlands, pp. 252-263.

Sager, S.M. (1960). On the transtadial transmission of insect viruses. Journal of Insect Pathology, 2, 307-309.

Salt, R.W. (1961). Principles of insect cold-hardiness. Annual Review of Entomology, 6, 55-74.

Schildmacher, H. (1950). Darmkanal und Verdauung bei Stechmückenlarven. Biologisches Zentralblatt, 69, 390-438.

Seecof, R.L. (1969). Sigma virus multiplication in whole-animal culture of Drosophila. Virology, 38, 134-139.

Sellke, K. (1936). Biologische und morphologische Studien schädlichen Wiesenschnaken (Tipulidae, Dipt.). Zeitschrift für wissenschaftliche Zoologie, 148, 465-555.

Shapiro, M. and Ignoffo, C.M. (1969). Nuclear polyhedrosis of Heliothis: stability and relative infectivity of virions. Journal of Invertebrate Pathology, 14, 130-134.

Smirnoff, W.A. (1961). A virus disease of Neodiprion swainei Middleton. Journal of Insect Pathology, 3, 29-46.

Smirnova, N.I. (1966). Vydelenie : kul'tivirovanie virusa meshotchatogo rasploda pchei (Isolation and cultivation of the sacbrood virus). Voprosy veterinarnoi virusologii (Problems of Veterinary Virology,) 2, 118-123. (Biological Abstracts (1967), 48, no. 91759).

Smith, C.E.G. and Westgarth, D.R. (1957). The use of survival time in the analysis of neutralisation tests for serum antibody surveys. Journal of Hygiene, Cambridge, 55, 224-238.

Smith, K.M. (1955). Intranuclear changes in the polyhedrosis of Tipula paludosa (Meig.). Parasitology, 45, 482-487.

Smith, K.M. (1958a). Early stages of infection with the Tipula iridescent virus. Nature, 181, 966-967.

Smith, K.M. (1958b). A study of the early stages of infection with the Tipula iridescent virus. Parasitology, 48, 459-462.

Smith, K.M. (1963). The arthropod viruses. in 'Viruses, Nucleic Acids and Cancer', ed. Dmochowski, L., pp. 72-84. Williams and Wilkins, Baltimore, Maryland.

Smith, K.M. (1967). 'Insect Virology', Academic Press, New York and London.

Smith, K.M. and Hills, G.J. (1959). Further studies on the electron microscopy of the Tipula iridescent virus. Journal of Molecular Biology, 1, 277-280.

Smith, K.M. and Hills, G.J. (1962). Multiplication and ultrastructure of insect viruses. Proceedings of 11th International Congress on Entomology, Vienna, 1960. Vol. 2, pp. 823-827.

Smith, K.M. and Rivers, C.F. (1956). Some viruses affecting insects of economic importance. Parasitology, 46, 235-242.

Smith, K.M. and Rivers, C.F. (1959). Cross-inoculation studies with the Tipula iridescent virus. Virology, 9, 140-141.

Smith, K.M. and Williams, R.C. (1958). Insect viruses and their structure. Endeavour, 17, 12-21.

Smith, K.M., Hills, G.J. and Rivers, C.F. (1961). Studies on the cross-inoculation of the Tipula iridescent virus. Virology, 13, 233-241.

Stairs, G.R. (1965a). Dosage-mortality response of Galleria mellonella (Linnaeus) to a nuclear-polyhedrosis virus. Journal of Invertebrate Pathology, 7, 5-9.

Stairs, G.R. (1965b). The effect of metamorphosis on nuclear-polyhedrosis virus infection in certain Lepidoptera. Canadian Journal of Microbiology, 11, 509-512.

Stairs, G.R. (1965c). Quantitative differences in susceptibility to nuclear-polyhedrosis virus among larval instars of the forest tent caterpillar, Malacosoma disstria (Hübner). Journal of Invertebrate Pathology, 7, 427-429.

Stairs, G.R. (1968). Inclusion-type insect viruses. in 'Insect Viruses', ed. Karamorosch, K., Current Topics in Microbiology and Immunology, 42, 129-167.

Steinhaus, E.A. (1947). A new disease of the variegated cutworm, Peridroma margaritosa (Haw.). Science, 106, 323.

Steinhaus, E.A. (1951). Report on diagnosis of diseased insects 1944-1950. Hilgardia, 20, 629-671.

Steinhaus, E.A. (1958). Crowding as a possible stress factor in insect disease. Ecology, 39, 503-514.

Steinhaus, E.A. (1960). The duration of viability and infectivity of certain insect pathogens. Journal of Insect Pathology, 2, 225-229.

Steinhaus, E.A. and Dineen, J.P. (1960). Observations on the role of stress in a granulosis of the variegated cutworm. Journal of Insect Pathology, 2, 55-65.

Steinhaus, E.A. and Leutenegger, R. (1963). Icosahedral virus from a scarab (Sericesthis). Journal of Insect Pathology, 5, 266-270.

Steinhaus, E.A. and Marsh, G.A. (1962). Report of diagnoses of diseased insects 1951-1961. Hilgardia, 33, 349-479.

Stewart, K.M. (1969). Studies on the biology of British crane-flies (Tipulidae, Diptera). Ph.D. Thesis, University of Glasgow.

Stevenson, J.P. (1959). Epizootiology of a disease of the desert locust, Schistocerca gregaria (Forskål), caused by nonchromogenic strains of Serratia marcescens Bizio. Journal of Insect Pathology, 1, 232-244.

Stoltz, D.B., Hilsenhoff, W.L. and Stich, H.F. (1968). A virus disease in Chironomus plumosus. Journal of Invertebrate Pathology, 12, 118-128.

Summers, M.D. (1969). Apparent in vivo pathway of granulosis virus invasion and infection. Journal of Virology, 4, 188-190.

Swaine, G. (1966). Generation-to-generation passage of the nuclear polyhedral virus of Spodoptera exempta (Wlk.). Nature, 210, 1053-1054.

Sylvester, E.S., Richardson, J. and Behncken, G.M. (1970). Effect of dosage on the incubation period of sowthistle yellow vein virus in the aphid Hyperomyzus lactucae. Virology, 40, 590-594.

Tanada, Y. (1953). Description and characteristics of a granulosis virus of the imported cabbageworm. Proceedings of the Hawaiian Entomological Society, 15, 235-260.

Tanada, Y. (1954). A polyhedrosis virus of the imported cabbageworm and its relation to a polyhedrosis virus of the alfalfa caterpillar. Annals of the Entomological Society of America, 47, 553-574.

Tanada, Y. and Chang, G.Y. (1960). A cytoplasmic polyhedrosis of the armyworm, Pseudaletia unipuncta (Haworth) (Lepidoptera, Noctuidae). Journal of Insect Pathology, 2, 201-208.

Tanada, Y. and Tanabe, A.M. (1965). Resistance of Galleria mellonella (Linnaeus) to the Tipula iridescent virus at high temperatures. Journal of Invertebrate Pathology, 7, 184-188.

Tanada, Y., Tanabe, A.M. and Reiner, C.E. (1964). Survey of the presence of a cytoplasmic-polyhedrosis virus in field populations of the alfalfa caterpillar, Colias eurytheme Boisduval, in California. Journal of Insect Pathology, 6, 439-447.

Thomas, R.S. (1961). The chemical composition and particle weight of Tipula iridescent virus. Virology, 14, 240-252.

Thompson, C.G. (1959). Thermal inhibition of certain polyhedrosis virus diseases. Journal of Insect Pathology, 1, 189-190.

Thompson, C.G. and Steinhaus, E.A. (1950). Further tests using a polyhedrosis virus to control the alfalfa caterpillar. Hilgardia, 19, 411-445.

Thompson, H.W. (1926). Leatherjackets and their control. Welsh Journal of Agriculture, 2, 228-233.

Tojo, S. and Kodama, T. (1968). Purification and some properties of Chilo iridescent virus. Journal of Invertebrate Pathology, 12, 66-72.

Tremaine, J.H. and Goldsack, D.E. (1968). The structure of regular viruses in relation to their subunit amino acid composition. Virology, 35, 227-237.

Utida, S. (1957). Cyclic fluctuations of population density intrinsic to the host-parasite system. Ecology, 38, 442-449.

Vago, C. (1968). Non-inclusion virus diseases of invertebrates. in 'Current topics in microbiology and immunology, Volume 42, Insect Viruses' ed. K. Maramorosch, pp. 24-37. Springer-Verlag, Berlin, Heidelberg, New York.

Vail, P.V. and Hall, I.M. (1969a). The influence of infections of nuclear-polyhedrosis virus on adult cabbage loopers and their progeny. Journal of Invertebrate Pathology, 13, 358-370.

Vail, P.V. and Hall, I.M. (1969b). Susceptibility of the pupa of the cabbage looper, Trichoplusia ni, to nucleopolyhedrosis virus. I. General responses. Journal of Invertebrate Pathology, 14, 227-236.

Vail, P.V., Hall, I.M. and Gough, D. (1969). Influence of a cytoplasmic polyhedrosis on various developmental stages of the cabbage looper. Journal of Invertebrate Pathology, 14, 237-244.

Van der Lann, P.A. and Wassink, H.J.M. (1969). On the capacity of Bacillus thuringiensis to spread in insect populations. Netherlands Journal of Plant Pathology, 75, 105-108.

Ver, B.A., Melnick, J.L., and Wallis, C. (1968). Efficient filtration and sizing of viruses with membrane filters. Journal of Virology, 2, 21-25.

Vickerman, K. (1960). Herpetomonas ludwigi (Kramář, 1950) n. comb., the trypanosomatid parasite of cranefly larvae (Diptera, Tipulidae). Parasitology, 50, 351-363.

Weiser, J. (1965). A new virus infection of mosquito larvae. Bulletin of the World Health Organisation, 33, 586-588.

Weiser, J. (1968). Iridescent virus from the blackfly Simulium ornatum Meigen in Czechoslovakia. Journal of Invertebrate Pathology, 12, 36-39.

Wellington, W.G. (1962). Population quality and the maintenance of nuclear polyhedrosis between outbreaks of Malacosoma pluviale (Dyar). Journal of Insect Pathology, 4, 285-305.

Whitcomb, R.F., Jensen, D.D. and Richardson, J. (1966). The infection of leafhoppers by Western X-disease virus. II. Fluctuation of virus concentration in the hemolymph after injection. Virology, 28, 454-458.

White, J.H. (1963). Observations on leatherjacket population assessment, forecasting and control measures. Proceedings of the second British Conference on Insecticides and Fungicides, Brighton, pp. 51-59.

White, J.H. (1966). Introductory review. Conference of Research Workers on Leatherjackets, ARC679/66.

White, J.H. (1967). The use of organochlorine insecticides and possible alternatives against leatherjackets. Plant Pathology, 16, 83-88.

White, J.H. (1968). Leatherjacket damage to grassland. Journal of the British Grassland Society, 23, 326-329.

W.H.O./F.A.O. (1969). Pesticide residues in food. World Health Organisation Technical Report Series, No. 417.

Wiemeyer, S.N. and Porter, R.D. (1970). DDE thins eggshells of captive American kestrels. Nature, 227, 737-738.

Wilkinson, A.T.S. and MacCarthey, H.R. (1967). The marsh crane fly, Tipula paludosa Mg., a new pest in British Columbia. Journal of the Entomological Society of British Columbia, 64, 29.

Williams, R.C. and Smith, K.M. (1957). A crystallisable insect virus. Nature, 179, 119-120.

Williams, R.C. and Smith, K.M. (1958). The polyhedral form of the Tipula iridescent virus. Biochimica et Biophysica Acta, 28, 464-469.

Willis, R.J. (1963). Another leatherjacket year? Agriculture in Northern Ireland, 37, 306-311.

Willis, R.J. (1965). Leatherjackets. Agriculture in Northern Ireland, 39, 388-389.

Wittig, G. (1963). Techniques in insect pathology. in 'Insect Pathology, an Advanced Treatise', ed. Steinhaus, E.A., Vol. 2, 591-636, Academic Pres

Woodard, D.B. and Chapman, H.C. (1968). Laboratory studies with the mosquito iridescent virus (MIV). Journal of Invertebrate Pathology, 11, 296-301.

Wrigley, N.G. (1969). An electron microscope study of the structure of Sericesthis iridescent virus. Journal of General Virology, 5, 123-134.

Wrigley, N.G. (1970). An electron microscope study of the structure of Tipula iridescent virus. Journal of General Virology, 6, 169-173.

Xeros, N. (1954). A second virus disease of the leatherjacket, Tipula paludosa. Nature, 174, 562.

Xeros, N. (1956). Insect virus diseases. Ph.D. thesis, Cambridge University.

Xeros, N. (1964a). Phagocytosis of virus in Tipula paludosa Meigen. Journal of Insect Pathology, 6, 225-236.

Xeros, N. (1964b). Development of the Tipula iridescent virus (TIV). Journal of Insect Pathology, 6, 261-283.

Younghusband, H.B. and Lee, P.E. (1969). Virus-cell studies of Tipula iridescent virus in Galleria mellonella (L.). 1. Electron microscopy of infection and synthesis of Tipula iridescent virus in hemocytes. Virology, 38, 247-254.

Younghusband, H.B. and Lee, P.E. (1970). Cytochemistry and autoradiography of Tipula iridescent virus in Galleria mellonella. Virology, 40, 757-760.

ADDENDUM TO REFERENCES

Burges, H.D. and Hussey, N.W. (1971), editors. 'Microbial control of insects and mites'. Academic Press, London and New York.

Chapman, H.C., Petersen, J.J., Woodard, D.B. and Clark, T.B. (1968). New records of parasites of Ceratopogonidae. Mosquito News, 28, 122-123.

Cunningham, J.C. (1970). The effect of storage on the nuclear polyhedrosis virus of the eastern hemlock looper, Lambdina fiscellaria fiscellaria (Lepidoptera : Geometridae). Journal of Invertebrate Pathology, 16, 352-356.

Cunningham, J.C. and Hayashi, Y. (1970). Replication of Chilo iridescent virus in Galleria mellonella : purification of the virus and the effect of actinomycin D and puromycin. Journal of Invertebrate Pathology, 16, 427-435.

Kalamakoff, J. and Robertson, J.S. (1970). Proceedings of the University of Otago Medical School, 48, 16-17.

Norris, J.R. (1971). Microbes as biological control agents. Symposium of the Society for General Microbiology, 21, 197-229.

Tinsley, T.W. and Kelly, D.C. (1970). An interim nomenclature system for the iridescent group of insect viruses. Journal of Invertebrate Pathology, 16, 470-472.