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Cricket Paralysis Virus, a Potential Control Agent for the Olive Fruit Fly, *Dacus oleae* Gmel.

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Representatives of several families of insect viruses were tested for growth and pathogenicity in the olive fruit fly, *Dacus oleae* Gmel. The viruses included nuclear polyhedrosis viruses, an iridovirus, two picornaviruses, and *Trichoplusia ni* small RNA virus (a member of the *Nudaurelia* β family), in addition to two naturally occurring viruses of the olive fruit fly. Two viruses, one of the two picornaviruses (cricket paralysis virus [CrPV] and the iridovirus (type 21 from *Heliothis armigera*), were found to replicate in adult flies. Flies which were fed on a solution containing CrPV for 1 day demonstrated a high mortality with 50% dying within 5 days and nearly 80% dying within 12 days of being fed. The virus was transmissible from infected to noninfected flies by fecal contamination. The CrPV which replicated in the infected flies was demonstrated to be the same as input virus by infection of *Drosophila melanogaster* cells and examination of the expressed viral proteins, immunoprecipitation of the virus purified from flies, and electrophoretic analysis of the structural proteins.

The olive fruit fly, *Dacus oleae* Gmelin, is the most serious pest of olives, causing early fruit drop, "sting" damage to table olives, and a reduction in quality and quantity of olive oil. Considerable resources are being expended on control and suppression protocols in many countries in the Mediterranean basin (4, 12; T. Manousis and N. F. Moore, Insect Sci. Appl., in press). The major controlling regimens involve the costly repeated application of chemical insecticides (13; T. Manousis and N. F. Moore, Sci. Ann. School Sci. Aristolelian Univ. Thessaloniki, in press). Although biological control procedures, such as the release of sterilized flies and the use of pheromone traps and *Bacillus thuringiensis*, have been attempted, no reports of pathogenic viruses exist. Laboratory-reared and wild populations of *D. oleae* in Greece were reported to contain two naturally occurring viruses. Their lethality was extremely low, and they did not appear to affect the normal generation of the flies (1; T. Manousis, S. M. Eley, J. S. K. Pullin, A. Lambropoulos, and N. F. Moore, Entomole Helle, in press). We approached the problem of finding a virus which would affect the flies first by using their endogenous viruses and second by using previously characterized insect viruses in the solution used to feed the flies. Because of the life cycle of the fly, which involves penetration of the fruit by the female to lay eggs, it is preferable not to attempt to infect larval stages of the insect (as is done in the vast majority of controlling regimens) but to infect the adults.

MATERIALS AND METHODS

Viruses. The following viruses were tested for their pathogenicity with adult *D. oleae* flies: *Heliothis armigera* (type 21) iridescent virus (3); *Drosophila* C virus (DCV) (8); cricket paralysis virus (CrPV), originally isolated from the Australian field cricket (10, 15); *Mamestra brassicae* nuclear polyhedrosis virus (NPV) (2); *Trichoplusia ni* NPV (7); and *T. ni* small RNA virus (9). In addition to these characterized virus preparations, viruslike particles purified from dead *D. oleae* larve collected from Northern Greece (Manousis et al., in press) and purified virus from a laboratory population of flies were tested for their effects on laboratory-maintained flies.

Insects. We used two stocks of *D. oleae* flies, which were kindly supplied as pupae by the Olive Institute, Corfu, Greece, and Benaki Phytopathological Institute, Kifissia, Athens, Greece. Groups of 40 to 60 1- to 2-day-old flies were transferred to sterilized containers which were then covered with nylon muslin and maintained at 23 ± 1°C at low humidity with a photoperiod of 12 h. Sterilized tap water was supplied on filter paper on a round plastic balcony 3 cm below the muslin cover. Food as described by Tischlinger (21) was supplied, and on day 1 it was diluted 2:1 with various virus preparations. Flies were transferred to clean containers to permit removal of excreta. Mortalities were recorded daily, and dead flies were frozen until examined for the presence of virus particles. Two control groups of flies were reared in parallel with experimental groups to permit evaluation of results.

Virus purification. All fractionation procedures were performed on ice or at 4°C. For control and test flies infected with CrPV, DCV, *T. ni* small RNA virus (a member of the *Nudaurelia* β family [11]), or viruslike particles isolated from *D. oleae* flies, dead or healthy individuals were macerated with a glass pestle and mortar containing 2 ml of 50 mM Tris hydrochloride (pH 7.2) for approximately 5 min. The homogenate was centrifuged for 10 min at 3,000 × *g*, and the supernatant from this step was centrifuged for 30 min at 12,000 × *g*. Virus particles were pelleted from this supernatant by centrifugation for 2 h at 107,000 × *g* over a pad of 15% sucrose in 50 mM Tris hydrochloride (pH 7.2). The resuspended pellet was overlaid on a linear 10 to 50% (wt/vol) sucrose gradient and centrifuged for 90 min at 150,000 × *g*. The gradient was divided into six aliquots, which were collected, diluted, and centrifuged for 3 h at 88,000 × *g*. Pellets were suspended and further purified on a second 10 to 50% (wt/vol) sucrose gradient. Purified virions from the different fractions were examined by electron microscopy.

Flies infected with NPVs were macerated as above, and the homogenate was centrifuged for 10 min at 1,000 × *g*. The supernatant was then centrifuged for 30 min at 12,000 × *g*, and the resultant suspended pellets were layered onto 50 to 60% (wt/wt) discontinuous gradients by centrifugation for 90

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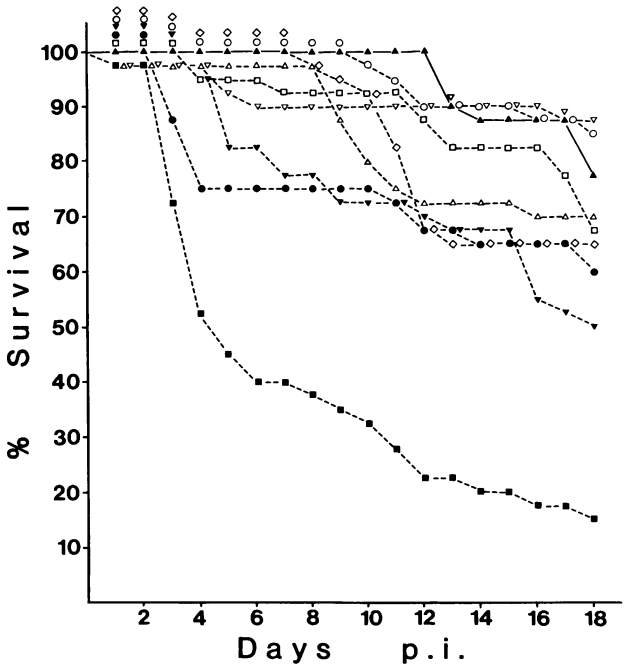

FIG. 1. Infectivity experiments on Benaki flies with DCV (▽), *T. ni* small RNA virus (○), *D. oleae* dead larvae (crude extract) (△), *M. brassicae* NPV (□), *D. oleae* virus isolate (◇), *T. ni* NPV (●), iridovirus (▼), CrPV (mean values of three experiments) (■), and on controls (mean values of two experiments) (▲).

min at 68,000 × *g*. The material at the interface was harvested, diluted, and pelleted for 30 min at 12,000 × *g*.

Flies infected with the iridovirus were macerated in 50 mM Tris hydrochloride (pH 7.2), and the resulting homogenate was centrifuged for 10 min at 3,000 × *g*. The supernatant was centrifuged for 1 h at 30,000 × *g*, and the pellet was suspended and layered onto a 15 to 40% (wt/vol) linear sucrose gradient and centrifuged for 1 h at 35,000 × *g*. The distinctive bluish-white band was collected, diluted, and pelleted for 1 h at 30,000 × *g*. The suspended pellet was layered onto a 40 to 80% (wt/vol) sucrose gradient in 50 mM Tris hydrochloride (pH 7.2) and centrifuged for 16 h at 35,000 × *g*. The band was concentrated and examined by electron microscopy.

Electron microscopy. Purified virus preparation were negatively stained with 2% uranyl acetate in distilled water and examined in a JEOL-100S electron microscope at an accelerating voltage of 100 kV.

Infection and radiolabeling of continuous cell line. *Drosophila melanogaster* cells were grown at 28°C in 25- or 75-cm^2 plastic flasks in Schneider *Drosophila* medium as previously described (10). Monolayers of cells were infected with various extracts and virus preparations by removal of the growth medium (Schneider *Drosophila* medium plus 10% fetal calf serum) from 25-cm^2 flasks and addition of the inoculum in 200 μl of maintenance medium (sterilized through 0.22-μm-pore-size filters). The cells were maintained for 1 h at 28°C and agitated every 20 min. The inoculum was removed, and cells were overlaid with 2 ml of Schneider *Drosophila* medium containing 2% fetal calf serum (maintenance medium) for a further 4 h at 28°C. Cells were washed with 2 ml of methionine-deficient Schneider *Drosophila* medium and starved for 25 min in 500 μl of the same medium. Intracellular proteins were radiolabeled by the addition of 100 μCi of [^{35}S]methionine for 1 h. Control and infected cells were prepared for polyacrylamide gel electrophoresis by the procedure of Tissieres et al. (22) and subsequently electrophoresed on 10 or 8 to 16% gradient gels by to the method of Studier (19).

RESULTS

Infectivity studies on flies. The flies in each container were allowed to feed on virus at the following concentrations: purified type 21 iridovirus 200 μg/ml; DCV, ca. 10^8 50% tissue culture infective doses per ml; CrPV, ca. 10^8 PFU/ml; *M. brassicae* NPV, 3 × 10^8 inclusion bodies per ml; *T. ni* NPV, 3 × 10^8 inclusion bodies per ml; and *T. ni* (*Nudaurelia* β type) small RNA virus, 250 μg/ml. The viruslike particles isolated from dead larvae collected in Northern Greece were at extremely low concentrations, and the small virus isolated from *D. oleae* flies was at a final concentration of 70 μg/ml. From the graph shown in Fig. 1, it is apparent that CrPV caused the highest mortality. Although the majority of control flies survived for several weeks (data not shown), more than 50 and 80% of the flies infected with CrPV were dead within 5 and 16 days, respectively. Infection with other viruses appeared to have a relatively minor effect; the most notable effect was that of the iridovirus by 18 days postinfection. However, considering that the flies were initially fed on concentrated virus particles or tissue culture fluids containing DCV and CrPV, it is to be expected that some deviation from the controls will occur. When the effect of CrPV on a different population of flies, those obtained from Corfu, was examined, the mortalities followed a similar pattern to that found with the flies from the Benaki Institute (Fig. 2). Hence there appears to be no strong synergistic effect between the different amounts of endogenous viruslike particles in the two populations and the CrPV used to infect them. To investigate whether the flies were killed when a smaller amount of virus was used to infect them, we used the same infection conditions of 1 day of exposure to food

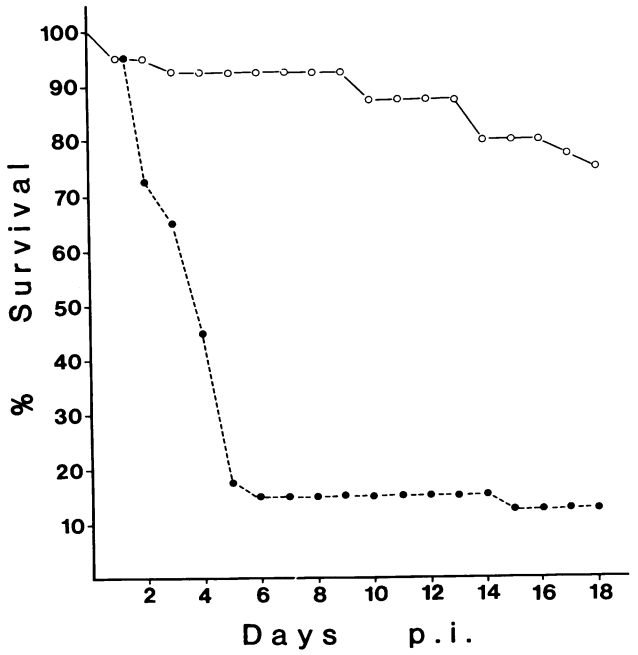

FIG. 2. Infectivity experiments with CrPV on flies from Corfu (●) and uninfected control flies (○).

containing virus, but CrPV was present at concentrations of 10^6 and 10^5 PFU (Fig. 3). The mortality of the flies does appear to be inoculum concentration dependent, at least in the earlier stages of infection (up to 18 days) (Fig. 3). If all the flies are taking an equal volume (10 μl) of food during the 1 day of infection, they are possibly being infected with an excess of 1,000 particles. Obviously, it would be valuable to evaluate the effect of virus concentrations over several generations of a stable insect population to see whether the mortality increases with time at low concentrations of input virus.

Since a low initial mortality was observed with smaller amounts of input virus, it was desirable to see whether the virus could be transmitted from fly to fly as a potential method for virus spread to increase the mortality in the tested populations. Three male and three female 1-day-old flies were permitted to feed on CrPV (10^8 PFU/ml). The flies were marked and introduced into a population of 40 flies. Unmarked flies were scored and harvested (Fig. 4). It appears that the virus is readily transmissible from one fly to another. The cross-infection could be occurring by contact, for example during mating, or, more likely, by the fecal-oral route (see below).

Progeny virus. Purification and electron microscopic examination of virus from flies permitted to feed on the different virus preparations were performed to determine whether any of the virus particles were replicating in the insects without causing mortality. Virions of the size expected for CrPV and the iridovirus were the only particles produced in large amounts (Fig. 5). Examination of the various low- and high-speed-fractionated preparations from the NPV-infected flies failed to reveal any occluded or nonoccluded virus particles. Relatively small numbers of particles were identified in the flies infected with the virus-like particles found in dead larvae in Northern Greece or

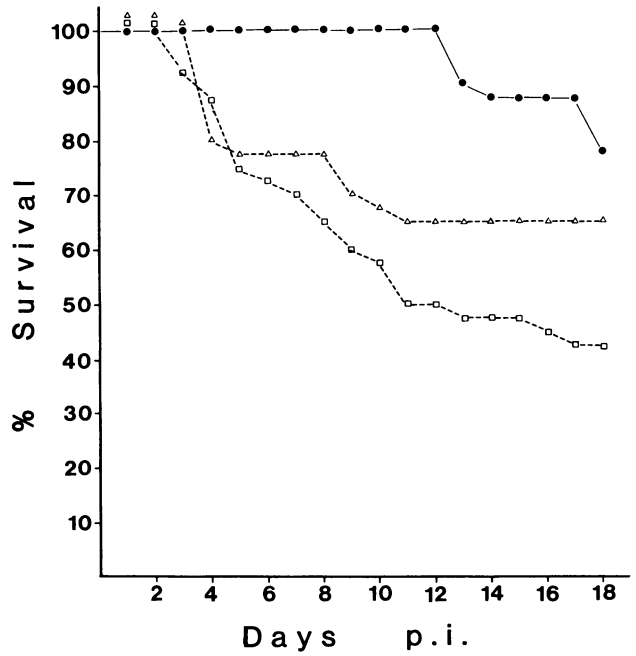

FIG. 3. Infectivity experiments on Benaki Institute flies with diluted original CrPV inoculum. Symbols: □, 100-dilution; △, 1,000-fold dilution; ●, control (mean values of two experiments).

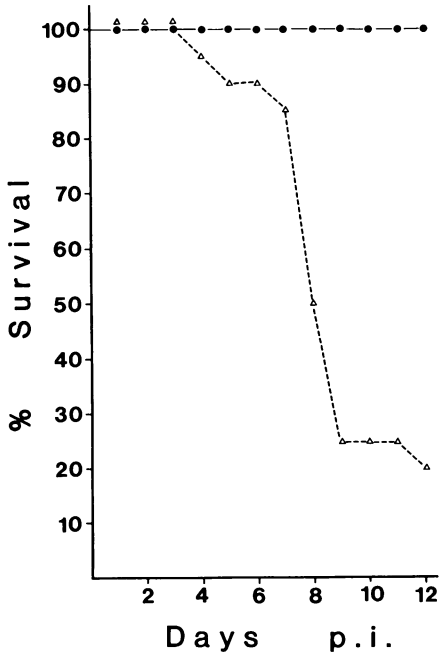

FIG. 4. Cross-infected flies (△) and uninfected flies (●).

with the virus from the inapparent infection of *D. oleae* (Fig. 5). It was hoped that it would be possible to elevate the concentration of the latter virus to make it more pathogenic.

Transmission of virus. To investigate whether the progeny virus was being transmitted in the feces, containers which held the infected flies were washed out with Schneider *Drosophila* medium, which was filter sterilized and used to infect *D. melanogaster* cells. The very distinctive cytopathic effect caused by CrPV was readily detected within 24 h, and the virions purified from the *D. melanogaster* cells appeared to be the same as CrPV by electron microscopy (Fig. 6).

Characterization of CrPV purified from infected *D. oleae* flies. The absence of large numbers of small virus particles the size of CrPV in other preparations suggests that the virus purified from the dead flies is indeed CrPV and not an inapparent virus induced by the particular infection conditions. However, there have been several reports of inapparent infections with both occluded and nonoccluded viruses, and to ensure that the virus causing mortality in the flies was CrPV, we performed several further experiments. Input virus and virus isolated from CrPV-infected dead flies and feces was used to infect *D. melanogaster* cells, which were then pulsed with [^{35}S]methionine. The radiolabeled proteins were separated by polyacrylamide gel electrophoresis, and the structural and precursor proteins associated with a CrPV infection were identified in the *D. melanogaster* cells infected by CrPV from dead flies and feces (Fig. 7). These are readily comparable to the induced proteins found in radiolabeled cells infected with input virus. When the virus purified from dead CrPV-infected flies was reacted with antiserum raised against pure CrPV, there was a strong line of identity (Fig. 8). However, there is still a possibility that CrPV was playing a minor role in the infection procedure with a second as yet unidentified small RNA virus which was initially present as an inapparent infection. To eliminate this possibility, we examined the purified virus proteins on a polyacrylamide gel and found that the only proteins identifiable were those associated with CrPV (Fig. 9). We would

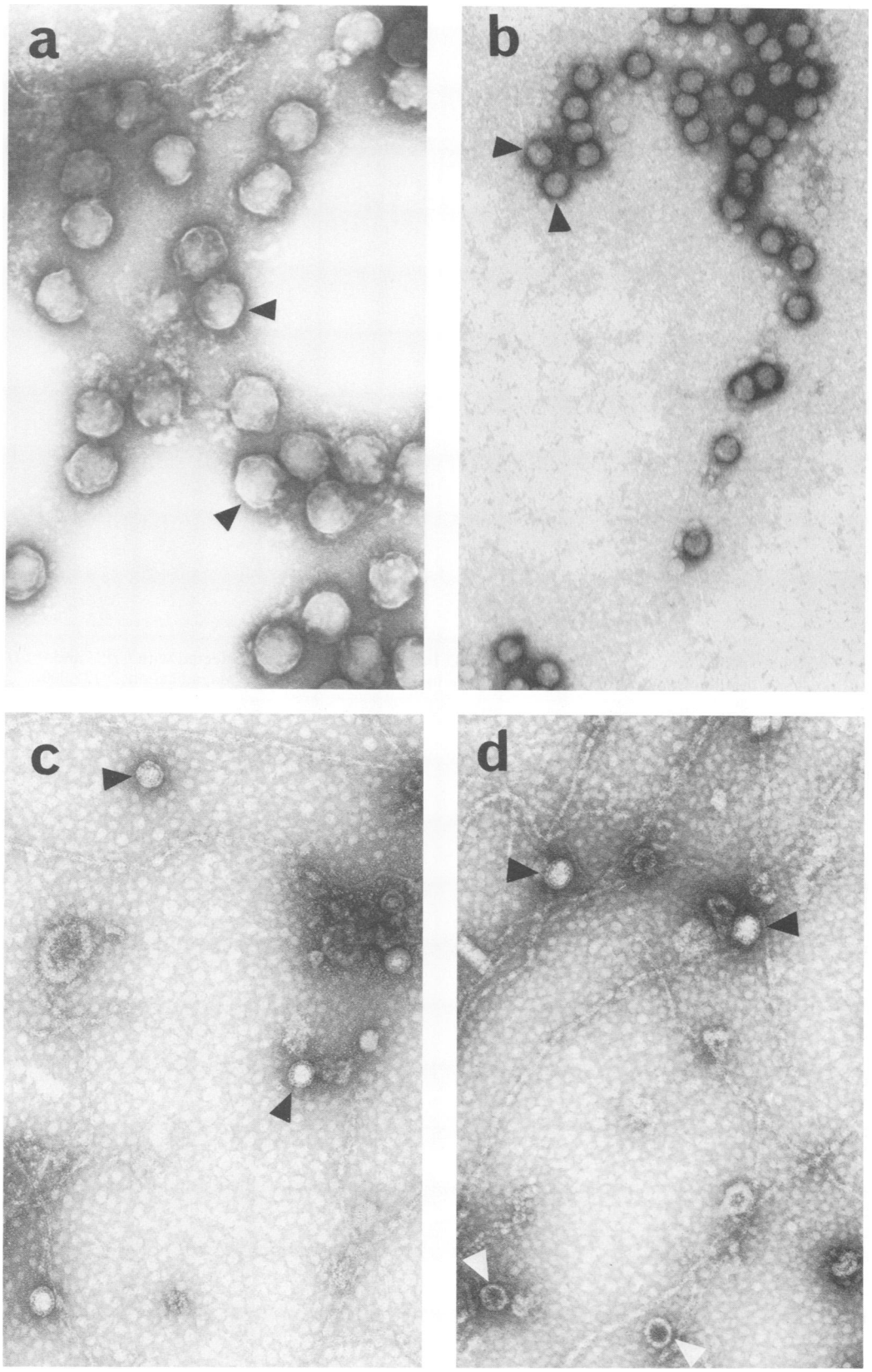

FIG. 5. Electron micrographs of particles isolated from Benaki Institute dead flies inoculated with (a) iridovirus, (b) CrPV, (c) *D. oleae* dead larva crude extract, and (d) *D. oleae* virus isolate. Black arrows indicate intact particles; white arrows indicate empty particles. Magnification, ×125,000.

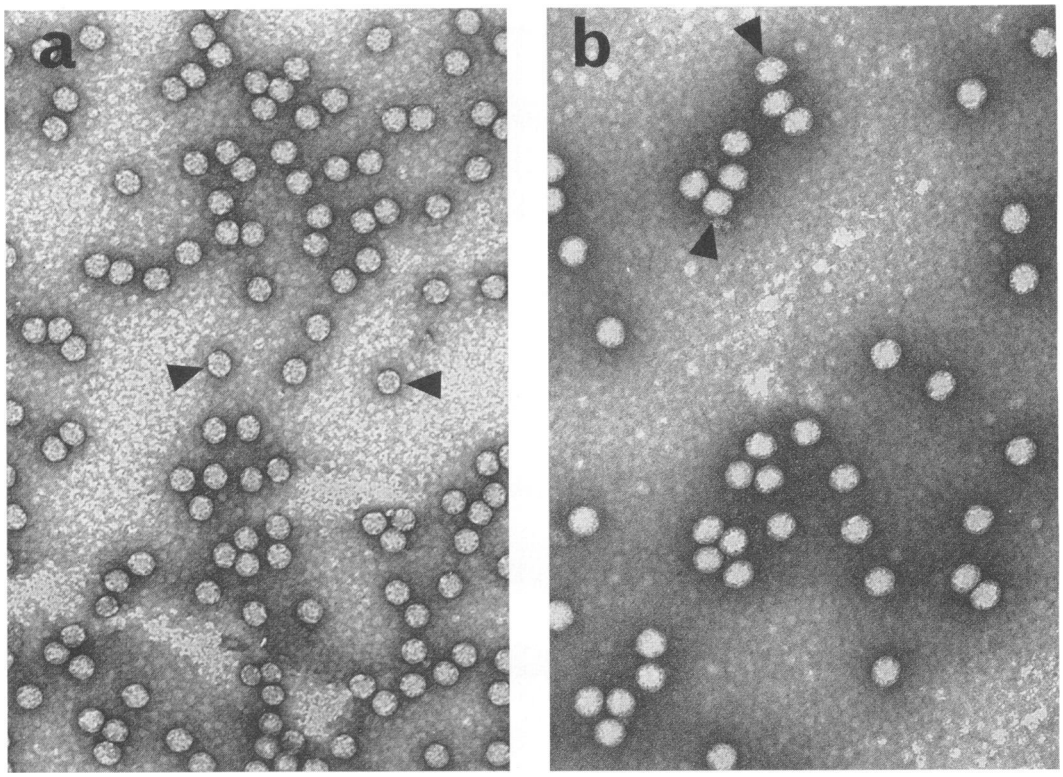

FIG. 6. Electron micrographs of particles isolated from (a) Benaki Institute dead flies cross-infected with CrPV and (b) *D. melanogaster* cells inoculated with feces of flies infected with CrPV. Black arrows indicate typical particles. Magnification, ×125,000.

have expected multiple protein bands if other small RNA viruses were apparent in the purified virus preparations.

DISCUSSION

CrPV is effective in causing a high mortality in laboratory-maintained colonies of *D. oleae* flies. Since it caused 50% mortality of adult flies within 5 days, the virus is potentially a potent control agent for *D. oleae*. A virus control agent lacks the immediacy of action found with a chemical pesticide or even a bacterial toxin, which cause fast mortality or paralyze the digestive system of the insect. Therefore, a virus such as CrPV will not prevent the immediate sting damage caused by females laying their eggs or prevent the successful generation of larvae. However, if it is possible to deliver the virus to a significant number of adult flies, it would serve to reduce the total population. The virus could act as an important component of an integrated pest management system. For example, the application of virus could be timed to coincide with the emergence of the adults. We have demonstrated that the virus can be transmitted and that the feces are the most likely route. The virus will than act on suceeding generations and even the same generation by fecal contamination of food and liquid sources.

The immediate problem is one of safety and of whether this type of virus should be used for field control of pests (5). Numerous studies have demonstrated that the structural proteins, genome size and arrangement, and replication events of CrPV resemble those associated with mammalian picornaviruses (for a review, see reference 11). However, there is no evidence that the insect picornaviruses replicate in species outside the Insecta. Some studies have demonstrated that there is a serological reaction of CrPV with the sera of cattle but this is probably attributable to exposure to the virus (17, 18, 20). There is also evidence to suggest a poor serological relationship between the mammalian picornavirus encephalomyocarditis virus and CrPV (20). There have been relatively few attempts to control pest species of insects with small RNA viruses. This is partly because relatively few natural epizootics have been identified and much of the research effort on control has been concentrated on baculoviruses and, to some extent, cytoplasmic polyhedrosis viruses. Effort has been concentrated on the occluded viruses because of the probably false supposition that these viruses are the ones which most readily survive the discontinuation of the insect life cycle. All insect viruses have to survive the discontinuities as well as evolve mechanisms to survive transmission from one insect to another. Many of the small RNA viruses appear to be associated with the gut and hence must be resistant to the digestive functions of the insect and be able to survive in the feces (which possibly acts to protect the virus against the major source of inactivation, i.e., UV light). The small RNAs can also obviously survive the proteases and nucleases released by decaying insect corpses. A picornavirus from *Gonometa podocarpi* was used to control this pest by being sprayed on exotic pines (primarily *Pinus patula*) in Uganda which were being defoliated by the larval stages of the insect (6). Another small RNA virus from the *Nudaurelia* β family, *Darma trima* virus, was used to control its homologous insect, a severe pest of oil and coconut palms in Malaysia (16). Both *G. podocarpi* and *Darma trima* were very effectively controlled by their homologous viruses.

If we can be convinced by further stringent safety testing

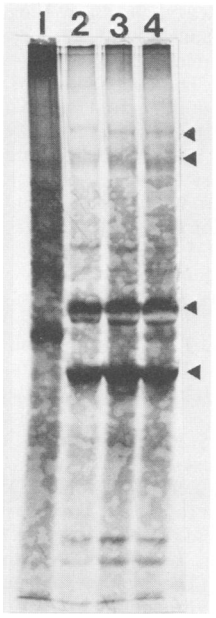

FIG. 7. Electrophoretic analysis of the [^{35}S]methionine-labeled polypeptides of infected and uninfected *D. melanogaster* cells which were pulsed at 28°C. Lanes: 1, control; 2, virus isolate from CrPV-infected dead flies; 3, feces of flies inoculated with CrPV; 4, CrPV original inoculum. CrPV intracellular proteins are indicated with arrows. Electrophoresis was in an 8 to 16% polyacrylamide gel, which was subsequently processed for autoradiography. The arrows show typical virus-induced proteins (see reference 10 for further details).

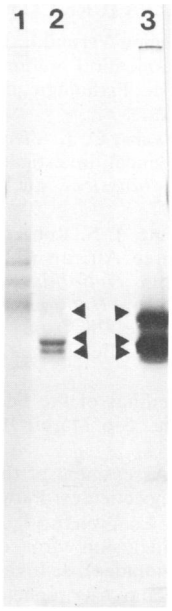

FIG. 9. Electrophoretic analysis of structural proteins of virus isolated from CrPV-infected dead flies (lane 2). Lane 1 shows extract of uninfected healthy flies, and lane 3 shows structural proteins of purified CrPV particles. Electrophoresis was in a 10% polyacrylamide gel, which was subsequently stained with Coomassie brilliant blue.

that CrPV does not infect man or animals, another aspect that must be examined is the host range of the virus in the environment in which it will be used. There are relatively few instances in which viruses which react with antiserum raised against CrPV have been identified from diseased insects in the field. However, Reinganum (14) transmitted CrPV experimentally to a range of insects within the Orthoptera and Lepidoptera. It is worth investigating whether the virus replicates in the naturally occurring insects in the region being sprayed. Particular emphasis should be placed on the effect of the virus on useful insects like bees and those involved in cross-pollination. However, it is also worth noting that chemical pesticides currently used are much less specific than any viruses. Little information is available on the worldwide occurrence of small RNA viruses such as CrPV, and it would be valuable to examine the viruses in the natural insect population by screening with antisera or cDNA probes.

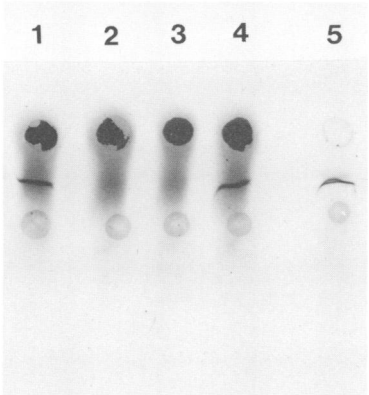

FIG. 8. Immunodiffusion test of CrPV (lane 5) and crude extracts of CrPV-infected dead flies (lanes 1 and 4) and uninfected flies (lanes 2 and 3) with hyperimmune antiserum to CrPV. The precipitin lines indicate the presence of CrPV antigens in the CrPV-infected flies.

Owing to the nature of the life cycle of *D. oleae*, it is unlikely that any biological insecticide can be targeted against the larval stages of the insect. Therefore, the virus must be aimed at the adult, and to do this it is not necessarily most effective to spray the trees with the virus by using handpacks or even crop-duster planes. It must be anticipated that the adults are attracted to baited areas containing active virus. The simplest baits would simply contain sweetened liquid, but a combination of color and pheromones could be used to make the feeding area more attractive. Much information is available on the color preferences of *D. oleae* and on the purification and characterization of the naturally occurring pheromones.

Obviously, it is important to attempt small field experiments or scale up laboratory work to investigate whether the use of CrPV can have a significant effect on a naturally occurring *D. oleae* population. It is imperative also to investigate (i) the possibility of virus inactivation under various conditions including UV light, (ii) the effective field dose, and (iii) optimal methods of introduction into the environment.

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