# CHARACTERIZATION OF A NUCLEAR POLYHEDROSIS VIRUS OF THE VARIEGATED CUTWORM, *PERIDROMA SAUCIA* (HÜBNER) (LEPIDOPTERA:NOCTUIDAE)

by

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# INTRODUCTION

Close to 200 virus diseases affecting invertebrates were reported in the 1950s (Hughes, 1957). Viruses of the Genus Baculovirus (Wildy, 1971), nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs), are being discovered in ever increasing numbers. Approximately 300 NPVs affecting Lepidoptera, Hymenoptera, and Diptera have been recorded in the literature (Harrap and Payne, 1979).

The Baculoviruses are considered to have the most potential for development and use as insecticides due to their lack of chemical, physical and biological properties in common with any known virus found in either vertebrates or plants (FAO/WHO, 1973). Due to the rapid development of strains of insect pests which are resistant to chemical insecticides, the use and exploitation of these viruses have received more attention. However, the lack of basic research has hindered the development of viral insecticides for without systems of identification, the usefulness of viruses and their ecological hazards cannot be tested satisfactorily.

A nuclear polyhedrosis virus of the variegated cutworm, Peridroma saucia (Hübner), was first observed in diseased variegated cutworms in an alfalfa field in Texas (Steinhaus, 1957). Martignoni (1964) studied the disease in the adult insect, and Harper (1970, 1971) conducted laboratory and field studies in Oregon utilizing the Steinhaus isolate. In June, 1977, diseased variegated cutworms were found feeding on wood

nettle (Laportea canadenis) in Sedgwick County, Kansas. Later in the season other diseased larvae were found in alfalfa fields in Riley County, Kansas. The causal agent of the disease was determined to be a nuclear polyhedrosis virus (Ramoska et al., 1978).

Presently, there is no research being done on the NPV of the variegated cutworm even though it is the most important cutworm on vegetables and is particularly damaging to tomatoes and potatoes. Since no studies to date have characterized this virus, investigation was directed primarily towards viral morphology, the infection process, biological activity and host-range studies. This research also examined the possible relationship between the Kansas and Steinhaus isolates. This was accomplished by comparing the polypeptide profiles of the polyhedrin obtained through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Since many recent reports have indicated the presence of an endogenous protease associated with the polyhedra of some NPVs, protease activity was examined in both NPV isolates studied. If a protease is present, inhibition of its activity is necessary for accurate comparisons of the structural polypeptides between the two isolates.

# MATERIALS AND METHODS

Rearing of variegated cutworm. Variegated cutworm larvae were reared individually in 1 ounce plastic cups (Thunderbird Container Corp., El Paso, Texas) on pinto bean diet (Reese et al., 1972). Four moths of each sex were placed in 1 gallon oviposition cages and fed an 8% sucrose solution. The eggs, which were laid on paper toweling, were surface sterilized with 0.5% NaOCL. The insects were maintained in a growth chamber on a 14-hour daylight regime at 22°C.

Sources of species used in host range studies. The southwestern corn borer, Diatraea grandiosella (Dyar), and European corn borer, Ostrinia nubilalis (Hübner), were obtained from Dr. Fred Poston, Department of Entomology, Kansas State University. Larvae of the indian meal moth, Plodia interpunctella (Hübner), were provided by Dr. Robert Mills, Department of Entomology, Kansas State University. All three of these species belong to the Family Pyralidae. The black cutworm, Agrotis ipsilon (Hufnagel), was supplied by Mr. Von Kaster at the USDA Corn Insects Laboratory, Ankeny, Iowa. Dr. Robert Burton, research entomologist, USDA, SEA-AR, at Oklahoma State University, provided the fall armyworm, Spodoptera frugiperda (J. E. Smith) and the corn earworm, Heliothis zea (Boddie). The black cutworm, fall armyworm, and corn earworm all belong to the Family Noctuidae. All insects were laboratory colonies maintained by the individuals

mentioned.

Sources of viral isolates. The Kansas isolate was purified from diseased insects found in Kansas in 1977. The Steinhaus isolate was supplied by Dr. J. D. Harper, Department of Zoology-Entomology, Auburn University.

Virus Purification. The stock preparation of polyhedral inclusion bodies (PIBs) was produced by mascerating artificially infected field-collected variegated cutworm larvae, filtering out the debris with glass wool and layering the liquid onto a discontinuous sucrose gradient ranging from 30% to 60% (w/v). The gradients were centrifuged for 2 hours at 28,000 rpm (Model PR-2, head #269, IEC, Boston, Massachusetts). The PIBs were rinsed in sterile distilled water, pelleted, and resuspended in water. The purified polyhedra were lyophilized and stored at 4°C. The Steinhaus isolate was lyophilized in 1967 and subsequently stored at 4°C. Upon examination of this material, host contamination and other debris could be seen.

Transmission electron microscopy (TEM) studies. Infected larval tissues were fixed in 5% glutaraldehyde in phosphate buffer, pH 7.2 for 2 hours and post-fixed in 4% osmium tetroxide at 4°C. Tissues were dehydrated by passage through a graded series of ethanol and embedded in Spurr's low viscosity resin. Tissue was sectioned on a Richert ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 at 60kv.

Scanning electron microscopy (SEM) studies. Preparation of the polyhedra for viewing in the scanning electron microscope included nebulizing them onto glass cover slips previously pasted onto SEM stubs with colloidal silver paste (Ted Pella, Tustin, California) and vacuum evaporating a thin layer of gold and palladium onto their surface. The polyhedra were viewed at 10kv with an Autoscan SEM.

Infectivity studies. The efficacy of the NPV was determined by per os bioassay of the variegated cutworm. Dosages of the standard viral preparation (described under virus purification) were quantitated by suspending 10 mg of polyhedra in 10 ml of water. This suspension was sonified at a setting of 4 on an ultrasonic sonifier (Model W185, Ultrasonics, Inc., Plainview, N.Y.) for three 1-minute intervals to break up any large aggregations and a hemocytometer was used to determine the quantity of polyhedra in the inoculum. Serial log 10 dilutions were made and 2.5 µl of each dilution was applied to a small amount of artificial diet in a 1 oz. plastic cup. Twenty cups were inoculated per dilution. One third or fourth instar larva was added to each cup and when it had completely consumed the viral-inoculated sample of diet, fresh diet was provided. An equal number of cups was inoculated with sterile distilled water as a control. Larvae in these infectivity tests were observed daily for mortality. Deaths due to NPV were confirmed by the presence of polyhedra in the tissue. These studies were

terminated 10 days after larval infection since all pupation or mortality had occured by that time. The percent mortality was computed as: the number of infected larvae divided by the total number of insects per dilution. The percent mortality was corrected using Abbott's Formula and all data were transformed and analyzed by the probit method (Finney, 1971).

Host range studies. All six lepidopteran species listed above were fed very high virus titer ($7.2 \times 10^6$ polyhedra) to detect any susceptibility to the variegated cutworm NPV.

Preparation of polyhedrin samples for sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE). A 10 mg aliquot of polyhedra in suspension was sonified and dissolved by the addition of 5 ml of .03 M $Na_2CO_3$ + .05 M NaCl (pH 11.1) for 90 minutes. This solution was passed through a .20 u Millipore filter (Millipore Corp., Bedford, Massachusetts) to remove the virus particles. A Diaflo Ultrafilter UM10 (Amicon, Lexington, Massachusetts) was employed to concentrate the protein. After concentration, the pH was lowered to 7.8 using .5 M Tris-Cl, pH 6.8. Similar samples were prepared with an additional step involving heating the polyhedral inclusion bodies to 65°C for two hours before carbonate treatment. This heat treatment was to insure inactivation of any proteolytic enzymes which might be present. Due to the higher amount of impurities in the Steinhaus isolate preparation, 20 mg suspended in 10 ml of water was treated similarly to the procedure outlined above for the Kansas isolate.

SDS-PAGE and molecular weight determinations. SDS-PAGE was carried out according to the procedures described by Laemmli (1970) with slight modifications. The sample buffer consisted of 0.0625 M Tris-Cl (pH 6.8), 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol and Bromophenol Blue. The system utilized was a vertical slab electrophoresis cell (Bio-Rad, Richmond, California).

Both heated and non-heated preparations of the Kansas and Steinhaus isolates were mixed with sample buffer in a 1:2 ratio. Samples were boiled 3 minutes before placing into 3% acrylamide stacking gel wells. Stacking was accomplished using 10 mA/slab for approximately two hours. When the dye front had entered the separating gel, consisting of a 4.5% to 20% acrylamide gradient, the current was increased to 30 mA/slab and allowed to run until the tracking dye reached the bottom of the gel. Slabs were treated with 5% Trichloroacetic Acid (TCA), stained with Coomassie Brilliant Blue (Eastman Kodak Co., Rochester, N.Y.), destained, dried under vacuum onto filter paper and then photographed.

The molecular weight of the polypeptide components was estimated by the procedure described by Weber and Osborn (1969). A plot of the relative mobility versus the log molecular weight of standards is linear; from the line obtained the molecular weight of the unknown polypeptides can be determined. Lasky (1978) has shown that gradient gels are generally superior to homogeneous gels. The following cali-

bration proteins were used: Lysozyme (14,300), β lactoglobulin (18,400), Trypsinogen (24,000), Pepsin (34,700), Albumin (45,000), bovine serum albumin (BSA) (66,000) and cross-linked BSA (132,000) (Sigma Chemical Co., St. Louis, Missouri).

# RESULTS AND DISCUSSION

Ultrastructure of the variegated cutworm NPV. The diseased host cells observed in thin sections were typical of those infected with a nuclear polyhedrosis virus (Harrap, 1972b; Steinhaus, 1963). In the early stages of infection the virions were seen associated with a virogenic stroma. Numerous enveloped and non-enveloped virions were present in the nucleoplasm closer to the periphery of the enlarged nucleus. Although both singly enveloped virions and multiply enveloped virions were observed; few singly enveloped virions were seen. The maximum number of virions/envelope noted was eight with the majority of the envelopes containing three or four virions. These results are similar to those obtained for Pterolocera amplicornia NPV in which not more than eight virions/envelope were observed (Day et al., 1958). The virions were rod-shaped and ranged in length from 270-430 mμ with a mean of 330 mμ (s.d.=.03, n=58). The mean width of the virions was 40 mμ ranging from 30-50 mμ (n=26). The mean virion length and width falls within the range of 200-400 mμ by 20-70 mμ reported for virions of other NPVs (Bellett, 1969; Heimpel, 1967). Only the enveloped virions were incorporated into the polyhedra which were observed developing in the host cell nucleus (Figure 1). A densely staining periphery around mature polyhedra was observed in transmission electron micrographs; this is the polyhedron membrane (Harrap, 1972a).

The polyhedral mean size was 1.54 μ (s.d.=.07, n=53) with a range of 1.0-2.13 μ (±5.0% accuracy). These measurements also fall within the ranges reported in the literature. Early reports indicated the size of the polyhedra may vary from 1.2-3.5 μ (Steinhaus, 1949). As more information was obtained, this size range was widened to include polyhedra measuring .5-15 μ (Bellett, 1969; Tinsley and Harrap, 1978). There is evidence that inclusion bodies of "strains" of NPV and GV have specific and consistent shape (Stairs, 1964; Vago et al., 1970). Figure 2 illustrates the size variation as well as the shape and surface texture of polyhedral inclusion bodies of the variegated cutworm.

When the variegated cutworm polyhedra were viewed under the SEM and phase contrast microscope, two size classes were detected. Extreme size variation was noted in Pseudaletia unipuncta where some polyhedra measured over 9 μ in diameter in unpurified suspensions (Tanada, 1959). Benz (1963) suggested that a correlation may exist between polyhedral size and the type of host cell infected since he observed that the smallest polyhedra were produced in the nervous tissue and the largest in the hypodermal cells. This relationship between type of tissue infected and size of polyhedra was not observed in the variegated cutworm NPV. Both large and small polyhedra could be seen in the fat body, tracheal epithelium and epidermal tissue. Polyhedra were observed in malpighian tubules and midgut epithelium, although the larger polyhedra were not.

The presence of the larger polyhedra in these tissues possibly may have been over looked due to a low number of them relative to the smaller polyhedra. A given cell did not produce both sizes of polyhedra; all polyhedra forming within a cell were similar in size.

Biological activity and host range of the variegated cutworm NPV. Per os bioassays were conducted to more closely simulate natural infectivity since in nature viral pathogens enter the insect host mainly through the mouthparts. The doses required to infect and cause death are much higher when the virus in introduced per os rather than when introduced directly into the hemocoel. This is due to the gut barrier which may play a role in the defensive reactions of the insect (Aizawa, 1962; Stairs, 1965).

The regression of polyhedra dose consumed per larva on probit mortality is depected in Graph 1. The $LD_{50}$ for third instar larvae was $2.6 \times 10^5$. The regression equation obtained was $Y=4.69 + .12X$ where Y is the probit mortality and X is the log polyhedral dose. The regression equation $Y=3.99 + .04X$ was obtained for fourth instar larvae, yielding an $LD_{50}$ of 2.4 x 106. Mortality data indicate that the earlier instar is more susceptible to the virus. Effective control of the variegated cutworm using this NPV would depend on early detection so viral application could be made while the cutworms were the most susceptible. No control larvae died from NPV in these infectivity studies.

Nuclear polyhedrosis viruses were once thought to be host specific but this idea has been negated by reports of NPVs capable of infecting other species in the same Family, in different Families and even in different Orders (Tompkins et al., 1969; Vail et al., 1971; Vail and Jay, 1973). The variegated cutworm NPV was tested on six other species of lepidopterans. Sixty individuals of each species was fed the virus and none of these insects died of a nuclear polyhedrosis viral infection. Earlier results (Ramoska et al., 1978) indicated that the black cutworm and army cutworm, Euxoa auxiliaris (Grote) were susceptible to the variegated cutworm NPV. However, the inoculum used and the bioassay parameters varied significantly from the present study. The army cutworm was not retested in this study and per os bioassays carried out on the black cutworm did not produce mortality. Additional species should be tested to further define the host range of this virus.

During the course of these studies, a cytoplasmic polyhedrosis virus (CPV) was observed in larvae obtained through field-collected females. This CPV was the first recorded affecting the variegated cutworm (Wilson and Ramoska, 1980).

Determination of protease activity. One method for determining the presence of a protease is to prepare the polyhedrin differentially so that conditions which permit protease activity can be compared to conditions inhibiting its activity. The cleavage of higher molecular weight polypeptides into polypeptides having lower molecular weights in indicative of protiolytic activity. Cibulsky et al. (1977) obtained multiple polypeptide bands for some polyhedrin and granulin; however the

conditions were conducive to protease activity. When some of these same viral isolates were tested under conditions inhibitive to protease, only a single polypeptide band was obtained (Summers and Smith, 1978). The first report of a protease was in the silkworm NPV (Yamafugi et al., 1957). Since this initial report, proteolytic enzymes have been found associated with the inclusion bodies of several NPVs and GVs (Eppstein et al., 1975; Harrap et al., 1977; Kozlov et al., 1975; McCarthy and Liu, 1976; Tweeten et al., 1978).

Proteases associated with NPVs and GVs have been found to be most active in alkaline pH with optimal activity occuring at a pH close to the gut pH of the larval host (Payne and Kalmokoff, 1978). Protease activity can be inhibited by heat and excessively high pH (Harrap et al., 1977). Inhibition of protease is also accomplished by the addition of the following chemicals to the carbonate solution: phenyl methyl sulfonyl fluoride (McCarthy and Liu, 1976); diisopropyl fluorophosphate (Summers and Smith, 1975); and the ions $Hg^{+2}$ or $Cu^{+2}$ (Eppstein and Thoma, 1975). Protease activity can be minimized by alkaline dissolution of the polyhedrin at 0°C (Summers and Smith, 1978) or by dissolving the polyhedrin in 67% acetic acid instead of carbonate (Harrap et al., 1977).

A study investigating the granulin of Pieris brassicae did not indicate the presence of a protease even under conditions favoring proteolytic activity (Brown et al., 1977). Zummer and Faulkner (1979) reported that an alkaline

protease is present in polyhedra grown in vivo but is absent from polyhedra propagated in tissue culture. This new information indicates that this particular protease is host contributed and the presence of an alkaline protease in the larval host should be investigated.

No differences in the polyhedrin profiles were observed between heated and unheated sample preparations in either one of the NPV isolates (Figure 3). Under the experimental procedures used in this study, protease activity was not demonstrated. The possibility exists that the variegated cutworm does not produce an alkaline protease which can become incorporated into the polyhedron during its formation or that the heat treatment used was not sufficient to denature the protease. Since protease activity has been investigated, more conclusive comparisons of the polypeptide profiles of the two NPV isolates can be made.

Comparison of polyhedrin profiles: Number of polypeptide bands and molecular weight determinations. Eight polypeptides, molecular weights ranging from 64,000 down to 14,000 daltons, were observed in Figure 3, tracks A and B, for the Kansas isolate. This same range of molecular weights was also indicated in the polypeptide profile of the Steinhaus isolate (Figure 3, tracks C and D) although nine polypeptides were identified. The Kansas and Steinhaus isolates have seven of the polypeptides in common but differ in three minor polypeptides. The Kansas isolate has a polypeptide (52,000 daltons)

which is lacking in the Steinhaus isolate and conversely the Steinhaus isolate has two minor polypeptides (45,000 and 43,000 daltons) which cannot be resolved in the polypeptide profile of the Kansas isolate. These small differences are reproducible but may be due to the varying age and/or purity of the virus stock preparations used.

The identification or separation of Baculoviruses based on the characteristics of their matrix proteins alone has been shown to be inadequate since these proteins all have molecular weights with a range of 25,000 to 31,000 daltons (Summers and Smith, 1976; Harrap et al., 1977; Summers and Smith, 1978). The major polypeptide components are the most intensely stained. The Kansas isolate had two major polypeptides with molecular weights of approximately 32,000 and 23,000 daltons. The 32,000 dalton polypeptide also stained intensely in the profile of the Steinhaus isolate but the 23,000 dalton polypeptide did not. This 32,000 dalton polypeptide has a higher molecular weight than any other NPVs reported in the literature. The inclusion body protein of the cytoplasmic polyhedrosis virus (CPV) of Nymphalis io has a major polypeptide with molecular weight of 37,000 daltons (Payne and Tinsley, 1974).

The polypeptide band near the top of all four sample tracks has twice the molecular weight of the major polypeptide. This 64,000 dalton polypeptide is most likely a dimer of the 32,000 dalton polypeptide since higher molecular weight

polypeptides are aggregates of the polypeptide monomers and consistently have twice the molecular weight as the monomer subunit (Summers and Smith, 1978).

The four lower molecular weight polypeptides common to both isolates consistently stained darker in the Steinhaus isolate's profile. Since no protease activity was indicated, these lower molecular weight polypeptides are assumed to be accurate and not breakdown products of larger polypeptides. If these profiles are accurate, this would indicate that the two variegated cutworm isolates are different.

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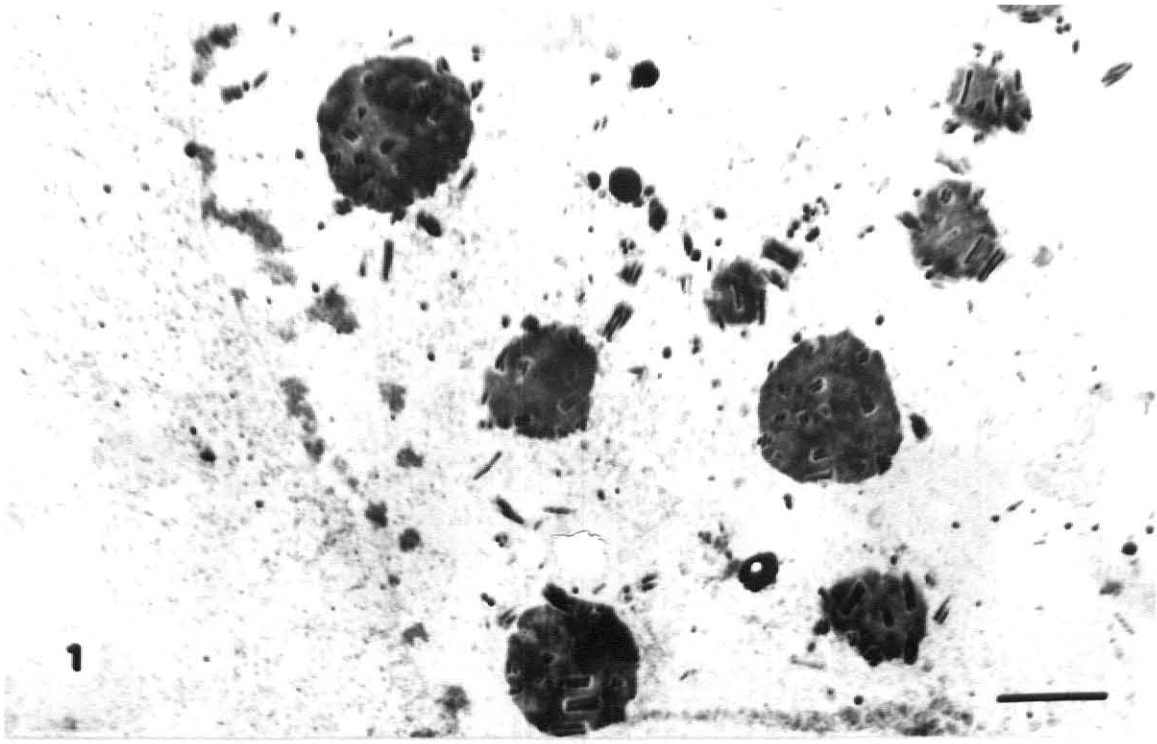
# CONCLUSION

The information presented here is just a starting point in the accumulation of knowledge that will be needed before this virus can be considered for use in controlling the variegated cutworm. More extensive bioassays and host range studies are needed as well as studies on the ecology of the virus, the possibility of producing it in tissue culture, and the hazards associated with its use.

If the two NPV isolates affecting the variegated cutworm are different, their biological activity needs to be compared to determine which one would be most suitable for formulating as a microbial insecticide. Further research can also be directed towards differences in polypeptide profiles of the virions, nucleic acid homology studies, and restriction endonuclease mapping of the viral genomes.

Fig. 1. Transmission electron micrograph of polyhedral inclusion bodies developing in the nucleus of the variegated cutworm cell. Bar=1 μ.

Fig. 2. Scanning electron micrograph illustrating the size variation, shape and surface characteristics of the polyhedra. Bar=1 μ.


1

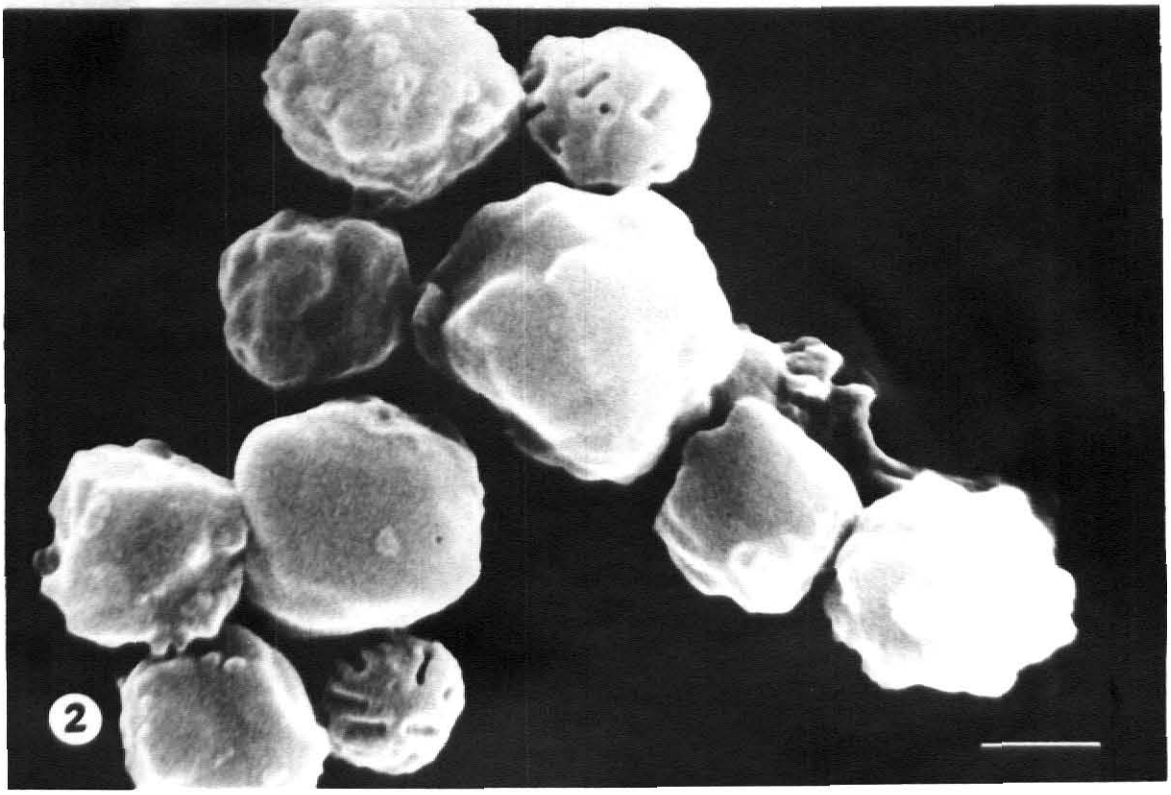
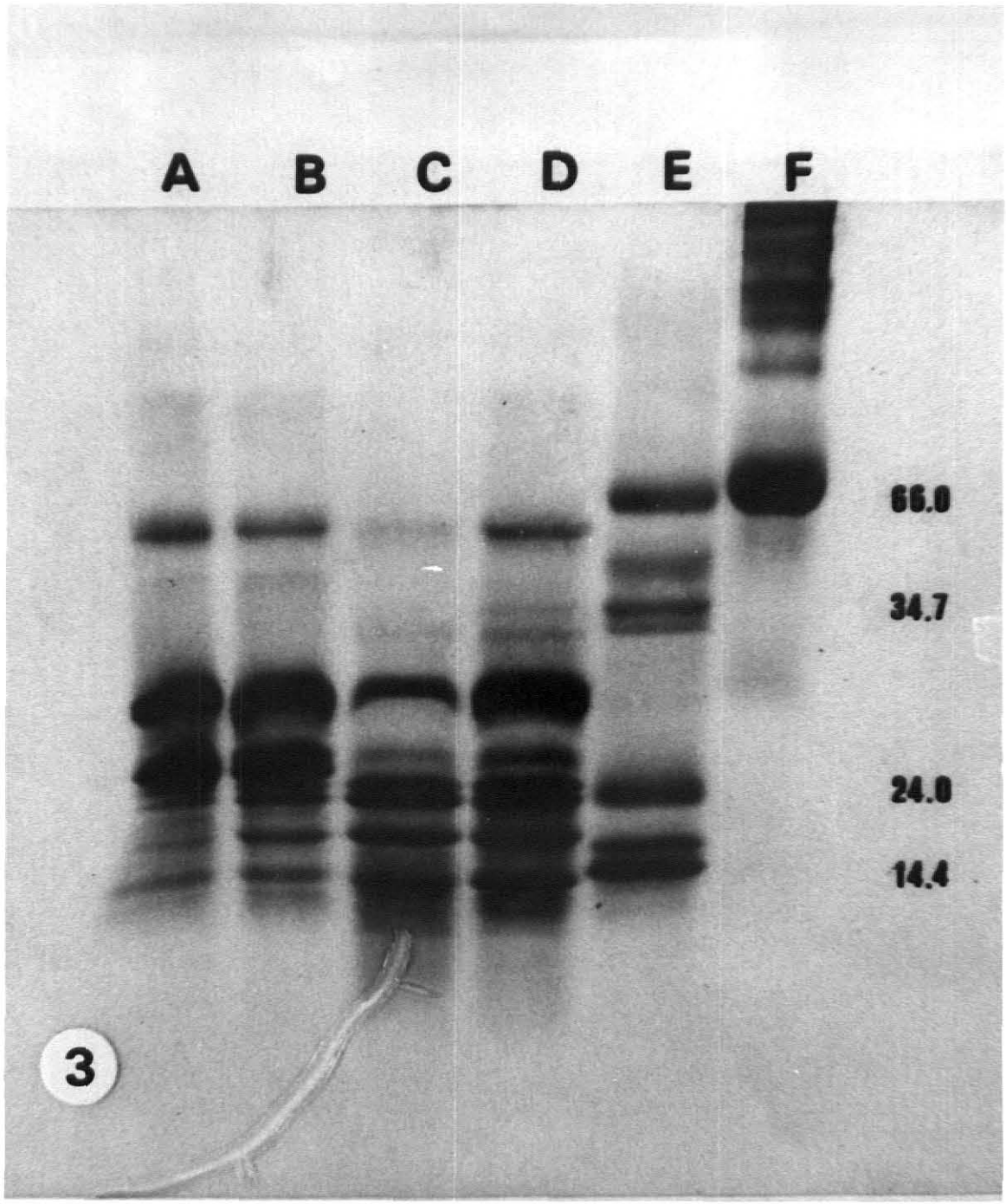
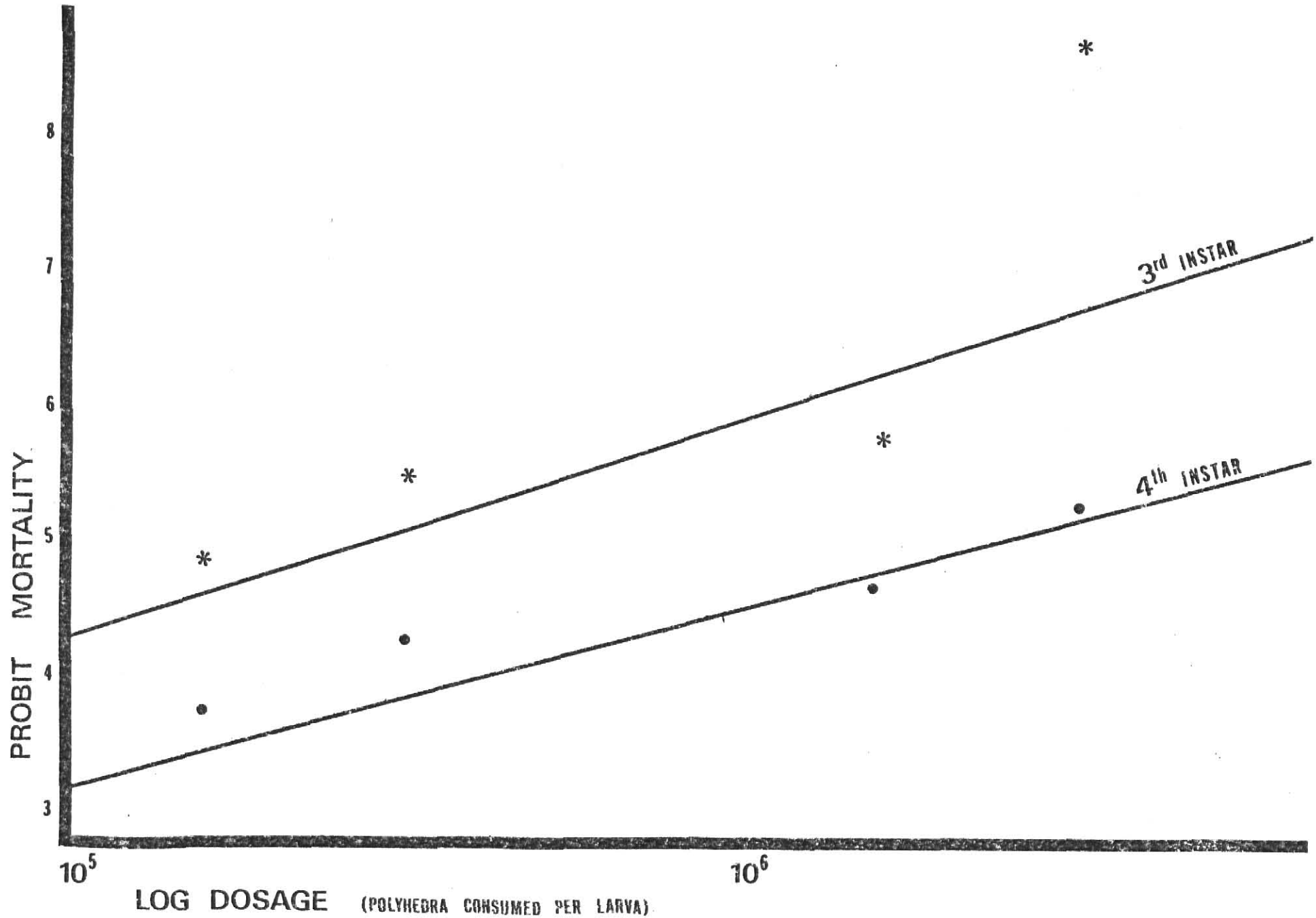

2

Fig. 3. Polypeptide profiles of the polyhedrin produced by SDS-PAGE. (A) Kansas isolate, heated; (B) Kansas isolate, unheated; (C) Steinhaus isolate, heated; (D) Steinhaus isolate, unheated; (E) Standard; (F) Cross-linked BSA.


A
B
C
D
E
F
66.0
34.7
24.0
14.4
3

Graph 1. Regression of polyhedra dose consumed per larva on probit mortality for third and fourth instar larvae.


8
7
6
5
4
3
PROBIT MORTALITY
3rd INSTAR
4th INSTAR
10^5
10^6
LOG DOSAGE (POLYHEDRA CONSUMED PER LARVA)

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# VITA

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# CHARACTERIZATION OF A NUCLEAR POLYHEDROSIS VIRUS OF THE VARIEGATED CUTWORM, *PERIDROMA SAUCIA* (HÜBNER) (LEPIDOPTERA:NOCTUIDAE)

by

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# ABSTRACT

The variegated cutworm, Peridroma saucia (Hubner) is an important vegetable pest. A nuclear polyhedrosis virus (NPV) affecting the variegated cutworm was first observed in Texas in 1953. In 1977, diseased variegated cutworms infected with a NPV were collected in Kansas. Neither of these two isolates have been characterized physically or biologically. This study was directed primarily towards characterization of the Kansas isolate. Biochemical studies of the two isolates were initiated in an attempt to determine whether they are identical.

The morphology of the variegated cutworm virus was examined by transmission and scanning electron microscopy. The polyhedra were observed in the nucleus of the larval cells and ranged in size from 1.0-2.13 μ in diameter. Virions ranged from 270-430 mμ in length and 30-50 mμ in width. Both singly enveloped virions and multiply enveloped virions were noted.

When the virus was tested on six other Lepidoptera, (Agrotis ipsilon (Hufnagel), Diatraea grandiosella (Dyar), Heliothis zea (Boddie), Ostrinia nubilalis (Hübner), Plodia interpunctella (Hübner), and Spodoptera frugiperda (J. E. Smith), none were affected indicating a narrow host range for this virus. The $LD_{50}$ was $2.6 \times 10^5$ and $2.4 \times 10^6$ for third and fourth instars, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the polyhedrin resulted in multiple bands with

the major polypeptide band having a molecular weight of 32,000 daltons. The polypeptide profiles of the two virus isolates were compared. Small reproducible differences in the profiles indicated that the two isolates were different. Protease activity was not found associated with the polyhedrin of either virus isolate under the experimental conditions used in this study.