Characterization of a Nuclear Polyhedrosis Virus Isolated from Diseased *Gonometa podocarpi* (Lepidoptera:Lasiocampidae)

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Gonometa podocarpi is an important pest of several species of pine in East Africa, and large numbers of trees in plantations in Kenya were partially or completely defoliated by the larval stage of this insect. After the infestation in the Mt. Elgon region, large numbers of dead and moribund larvae were found on the ground. Examination of extracts of these larvae demonstrated the presence of an occluded virus. Electron microscopy of purified sectioned polyhedra demonstrated the presence of virus particles containing from 1 to 12 nucleocapsids. Purification of virus particles from polyhedra was accomplished by using alkali solubilization and sucrose gradient centrifugation. Virus particles contained 15 proteins as determined by polyacrylamide gel electrophoresis. Detergent solubilization of the virus particles released polyhedra containing one major structural protein. Electron microscopy of purified virus particles and nucleocapsids demonstrated them to be similar in structure to previously recorded nuclear polyhedrosis viruses. The viral deoxyribonucleic acid was extracted and spread for electron microscopy and was determined to have a size of approximately 80×10^6 daltons.

The genus *Gonometa* occurs in many parts of Africa (2), but *Gonometa podocarpi* seems to be confined to East Africa (3) and was first recorded from Mt. Elgon, Kenya (1), attacking *Podocarpus* sp., an indigenous conifer. Since then, the insect has adapted to feeding on exotic softwoods, particularly *Cupressus lusitanica*, *Pinus radiata*, and *Pinus patula* (3).

Recent surveys of defoliating caterpillars in the Mt. Elgon areas revealed widespread disease in *G. podocarpi* attacking *P. patula*. It was of interest to determine the possible causal agent or agents of this high insect mortality. Harrap et al. (11) isolated a small ribonucleic acid virus from diseased *G. podocarpi* collected on *P. patula* in the Kigezi district in Uganda, and Longworth et al. (15) determined its properties. In this study, two viruses were isolated from the Kenyan insects, a nuclear polyhedrosis virus (NPV; Baculoviridae) and a small ribonucleic acid virus which was serologically related to the original Uganda isolate. Small ribonucleic acid viruses frequently cause mild or inapparent infections in insects, and so the widespread mor-

tality in the *G. podocarpi* larvae from the Saboti Forest in the Mt. Elgon region most probably arose from the NPV. This paper reports the partial characterization of this virus.

MATERIALS AND METHODS

Insects and virions. Dead and dying *G. podocarpi* larvae were collected from the forest floor. The larvae were in the later stages of development and had been feeding on *P. patula*. NPV polyhedra were purified from artificial infections in their homologous insects, *Spodoptera littoralis*, *Gilpinia hercyniae*, and *Heliothis zea* (12, 13).

Preparation of polyhedra. Larvae were homogenized in deionized water, and the suspension was filtered through two layers of cheesecloth to remove insect debris. Whenever possible, the polyhedra and virus particles were maintained at 4°C or on ice during the purification procedures to reduce melanization and virus degradation. The filtrate was centrifuged at $2,000 \times g$ for 30 min. The pellet from this centrifugation was resuspended, overlaid on pre-equilibrated 2% step 50 to 60% (wt/wt) sucrose gradients, and centrifuged at $87,000 \times g$ for 45 min. Polyhedra were harvested, diluted with distilled water, pelleted at $2,000 \times g$ for 30 min, and rebanded on a 50 to 60% (wt/vol) sucrose gradient.

Kinetics of polyhedra dissolution. Dissolution was monitored at different pH's by observing spectrophotometric changes at 400 nm after the addition of

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FIG. 1. Several sectioned polyhedra of *G. podocarpus* NPV.

variable amounts of 0.1 M sodium carbonate. The alkaline protease activity associated with the polyhedra was assessed by incubating the polyhedra at pH 10.7 (30°C) and removing aliquots at intervals. The aliquots were mixed with equal volumes of ice-cold 2 M sodium acetate (pH 4) for 20 min. The precipitate was pelleted at $2,000 \times g$ for 5 min and redissolved in 2% sodium dodecyl sulfate and 2% 2-mercaptoethanol in 10% electrophoresis buffer containing 15% glycerol and 0.1% bromophenol blue as a marker dye for electrophoresis.

Purification of virus particles from polyhedra. Suspensions of polyhedra were mixed with an equal volume of 0.1 M sodium carbonate and incubated for 10 min at 30°C. The clarified suspension was then overlaid on a 30 to 65% (wt/wt) sucrose gradient in 0.05 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.4) and centrifuged at $110,000 \times g$ for 60 min in a 6 $\times$ 16-ml swinging-bucket rotor. The virus bands were harvested, diluted, and pelleted at $110,000 \times g$ for 60 min.

Preparation of nucleocapsids from virus particles. A final concentration of 2% Nonidet P-40 was added to virus suspensions, and the mixture was incubated at 30°C for 30 min. The nucleocapsids were separated from the solubilized envelopes by pelleting through a 10% sucrose pad at $90,000 \times g$ for 1 h. The pellets were prepared for electron microscopy and polyacrylamide gel electrophoresis.

Electron microscopy. Polyhedra were fixed in 2%

(vol/vol) glutaraldehyde and 1% (wt/vol) osmium tetroxide as described by Harrap et al. (12). After they were embedded in Epikote resin, 80 to 100-nm-thick sections were cut and stained with uranyl acetate and lead citrate (12). Virus particles and nucleocapsids were negatively stained, using 2% uranyl acetate, pH 4 (7-10). Extracted viral deoxyribonucleic acid (DNA) was spread and prepared for electron microscopy, using a procedure previously described (4). All samples were examined with a JEOL 100CX electron microscope.

Isolation of viral DNA. The polyhedral suspension was dissolved, using an equal volume of 0.1 M Na_2CO_3 , and incubated for 30 min at 30°C. A final concentration of 2% *N*-lauryl sarcosine in 0.5 M ethylenediaminetetraacetate was added, and the mixture was incubated for 2 h at 60°C. The mixture was overlaid on an initial concentration of 54% (wt/vol) CsCl containing 0.5 μg of ethidium bromide per ml, overlaid with liquid paraffin, and centrifuged at $215,000 \times g$ for 18 h in a 16 $\times$ 14-ml swing-out rotor (MSE). The clearly visible DNA band (UV-254) was dialyzed against several changes of 100 volumes of deionized water for 3 days and extracted with an equal volume of propan-2-ol to remove the ethidium bromide before precipitation by the addition of an equal volume of ice-cold ethanol. The DNA was immediately concentrated by centrifugation at $2,000 \times g$. The DNA pellet was resuspended in a small volume of deionized water and kept at 4°C for further use.

Polyacrylamide gel electrophoresis. All protein samples were boiled for 2 min at 100°C before electrophoresis in a final concentration of 2% sodium dodecyl sulfate (wt/vol)–2% 2-mercaptoethanol (vol/vol)–15% (vol/vol) glycerol–0.001% (wt/vol) bromophenol blue tracking dye. Samples were boiled at 100°C for 30 s shortly before electrophoresis. Samples were electrophoresed on 16-cm-long polyacrylamide slab gels, using a discontinuous buffer system (14, 16), or on 10% polyacrylamide tube gels (9). Gels were stained in 1.0% Coomassie brilliant blue R250 in 45% methanol–7% acetic acid and also destained in the methanol-acetic acid solution.

RESULTS

Polyhedron structure. Electron microscopy of sectioned polyhedra demonstrated the presence of multi-enveloped virus particles within crystalline structures, as found with other NPVs. Figure 1 shows a typical field of polyhedra, several of which are partly degraded, which can probably be attributed to both the sectioning

and the relatively long time between field collection and freezing of the preparations. Some of the fragments observed in the preparation are probably outer parts of complete crystals cut off center. Enlargement of a polyhedron shows the presence of bundles of nucleocapsids within one virus envelope cut at various angles (Fig. 2). Bundles of up to 12 nucleocapsids were apparent within one virus envelope. In Fig. 2 transverse sections of virus particles containing one, two, and three nucleocapsids can be seen.

Whole purified polyhedra were solubilized in sodium dodecyl sulfate and 2-mercaptoethanol and electrophoresed on 10% polyacrylamide cylindrical gels and 12% slab gels. Comparison of the protein pattern with the polyhedra from the viruses from *S. littoralis* and *Gilpinia hercyniae* (channels 1 and 2, Fig. 3) demonstrated a similar pattern for the *G. podocarpus* NPV (channel 3, Fig. 3). There were large amounts of one protein with a molecular weight of approximately 30,000

FIG. 2. Sectioned polyhedron of *G. podocarpus* NPV. Bar, 400 nm.

FIG. 3. Polyacrylamide gel analysis of the polyhedral proteins from *S. littoralis* (1), *G. hercyniae* (2), and *G. podocarpus* (3). Arrow indicates the major polyhedral protein. Sodium dodecyl sulfate-solubilized and 2-mercaptoethanol-reduced samples were electrophoresed on parallel 10% cylindrical gels.

and many minor protein components. Purification of the polyhedra in a 0.1% sodium dodecyl sulfate solution (leaving the polyhedra apparently intact) removed many of the other bands (data not shown).

Dissolution of polyhedra. Initial attempts to release virus particles from the polyhedra by using the normal procedure of alkaline dissolution (9, 18, 19) proved unsuccessful.

Multinucleocapsid NPV should contain a large number of components which can be separated on sucrose gradients. As the gradients did not resolve virus particles or nucleocapsids after normal dissolution, it was probable that virus was degraded; this was attributed to a hyperactive alkaline protease. The dissolution of the polyhedra was monitored at 400 nm, using a range of alkaline pH, and the breakdown was

found to occur between pH 10.3 and 10.65 (Fig. 4). At higher pH the dissolution occurred faster. Comparison with the dissolution of *H. zea* polyhedra (data not shown) demonstrates that the dissolution of *G. podocarpus* occurred under less alkaline conditions. Very little dissolution of *H. zea* polyhedra occurred at pH 10.7, whereas at pH 10.65 a considerable number of the polyhedra of *G. podocarpus* were degraded. The rate of the *H. zea* dissolution was, however, much faster and the total dissolution went closer to completion. By 30 min the *G. podocarpus* polyhedra were dissolved to about 80% at high pH, whereas at high pH the *H. zea* polyhedra were dissolved to 90% within 5 min. Polyacrylamide gel electrophoresis of the residual polyhedra protein after dissolution (Fig. 5) showed that the major structural protein was being degraded very quickly, much of the reaction occurring within 5 min. The evidence suggested that, although fast degradation of the polyhedral protein was occurring, the virus particle degradation could not be attributed solely to the alkaline protease. To attempt to terminate the degradation of virus particles by other proteases, several procedures were used, including introduction of zinc ions or phenylmethylsulfonyl fluoride (PMSF) into the dissolution mixture and preheating of the polyhedral preparation before dissolution. A normal dissolution of polyhedra was obtained with pretreatment with PMSF or heat, and sucrose gradient analysis of the total

FIG. 4. Dissolution of *G. podocarpus* polyhedra under several alkaline conditions measured as a function of time. The pH's of the solutions were: (1) 10.3; (2) 10.65; (3) 10.7; (4) 10.9; and (5) 11.05. OD⁴⁰⁰, Optical density at 400 nm.

FIG. 5. Dissolution of the major protein from *G. podocarpi* polyhedra. The polyhedra were incubated at pH 10.7 (30°C), and aliquots were removed at the time intervals shown (the numbers refer to minutes). The residual protein was precipitated with ice-cold 2 M sodium acetate (pH 4) for 20 min and dissolved in a final concentration of 2% sodium dodecyl sulfate-2% S-mercaptoethanol-15% glycerol before electrophoresing in 10% polyacrylamide tube gels. The tube gels were stained with Coomassie brilliant blue R250, destained, and scanned on a Joyce-Loebl densitometer.

mixture demonstrated the presence of virus particles containing one to many nucleocapsids. With 1 mM PMSF present during dissolution, bands of virus particles were obtained (Fig. 6) which contained up to 10 nucleocapsids.

Virus particles and nucleocapsids. Virus

particles prepared in the presence of PMSF were examined by electron microscopy after negative staining (Fig. 7) and were found to have size, structure, and shape similar to those previously described for other preparations (9, 12, 19). Nucleocapsids were prepared by disruption with Nonidet P-40 and examined by negative staining and electron microscopy (Fig. 8). It was necessary to add PMSF to the detergent-treated virus preparations to prevent degradation, as the proteases were apparently inactivated by the earlier treatment during the polyhedral dissolution.

FIG. 6. Gradient analysis of virus particles after treatment of a polyhedral suspension with an equal volume of 0.1 M sodium carbonate. The 30 to 65% (wt/wt) sucrose gradients in 0.05 M tris(hydroxymethyl)aminomethane, pH 7.4, were centrifuged at $100,000 \times g$ for 60 min. Protease activity was inhibited with 1 mM PMSF during the dissolution. Eight major and two minor bands of multi-enveloped virus are apparent.

FIG. 7. *G. podocarpi* NPV virus particles. Bar, 200 nm.

FIG. 8. Nucleocapsids of *G. podocarpi* NPV. Bar, 100 nm.

The nucleocapsids prepared by the detergent treatment were similar to those previously reported for other NPV systems (10, 12).

The virus proteins prepared from preheated polyhedra and polyhedra dissolved in the presence of PMSF were examined by polyacrylamide gel electrophoresis. Figure 9 shows the proteins

from PMSF-prepared virus (channel A) and virus from polyhedra heated for 10 (channel B) and 2 (channel C) min electrophoresed on a 12.5% polyacrylamide slab gel. With the shorter heat treatment there is apparent residual degradation of a major virus structural protein. The two arrowed proteins are present in different proportions with the different methods of dissolution of the polyhedra. There was considerable variation in the small amounts of higher-molecular-weight polypeptides in the virus particles. Figure 10 shows a preparation of nucleocapsids (channel A) compared with the protein of virus particles. Fifteen structural proteins are indicated, but several other minor proteins were usually found in various amounts. The molecular weights of the proteins are given in Table 1. Nucleocapsids contained one major protein (no. 14) and a few minor components (indicated by arrows). The apparent degradation of the major structural protein (no. 14) of the nucleocapsid (apparently into protein 15) is the probable reason for the absence of nucleocapsids (or virus particles) from the gradients of the dissolved polyhedra which had not been pretreated with heat or PMSF.

DNA of the *G. podocarpi* NPV. The viral DNA was extracted and purified on CsCl gradients as described in Materials and Methods. A large number of molecules of DNA were examined in the electron microscope, and the majority were found to be circular. Figure 11 shows an example of a complete circle. The lengths of 17 molecules were measured and averaged. The

FIG. 9. Proteins of virus particles prepared from polyhedra heated at 60°C for 2 (C) and 10 (B) min. Channel A shows the proteins of virus particles prepared from polyhedra which were dissolved in the presence of a final concentration of 1 mM PMSF. All polyhedra preparations were mixed with an equal volume of 0.1 M Na_2CO_3 and incubated at 30°C for 10 min. The samples were then overlaid on 10 to 50% sucrose gradients in 50 mM tris(hydroxymethyl)aminomethane, pH 7.4, and centrifuged as described in the text.

molecular weight of the DNA was found to be 80.22×10^6 , with a variation of 6.79%.

DISCUSSION

The NPV isolated from *G. podocarp*i has a polyhedra structure, virus particles, and nucleocapsids similar to those described for other virus systems of this type. The preparation of virus particles from the nucleocapsids was hindered by the presence of proteases associated with the polyhedra. However, by using polyhedra which

were heated before dissolution or incubated in the presence of 1 mM PMSF during dissolution, it was possible to prepare virus particles and nucleocapsids. The virus particle and nucleocapsid degradation could not be attributed to the alkaline protease as it would be self-defeating for virus proliferation if a virus-coded (or selected) enzyme was causing virus destruction. The nucleocapsids contained one major and at least two minor proteins, whereas the virus particles contained a large number of proteins associated with their envelopes. This is similar to other NPVs which contain one or two and several proteins associated with their nucleocapsids (12, 18, 19). The average molecular weight of the

FIG. 10. Proteins of nucleocapsids (A) and virus particles (B) electrophoresed on 12.5% polyacrylamide slab gels. The nucleocapsid proteins are indicated by arrows, and the virus proteins are numbered from 1 to 15 (see Table 1).

FIG. 11. Electron micrograph of a typical molecule of circular DNA from *G. podocarpi* NPV. Purified DNA was spread and shadowed as previously described (5).

TABLE 1. Molecular weight of the proteins of *G. podocarpi* NPV

Protein no. ^a	Mol wt ($\times 10^{-3}$) ^b
1	108
2	97
3	90
4	82
5	77
6	75
7	62
8	56
9	54
10	43
11	42
12	40
13	39
14	35
15	33

^a See Fig. 10 for protein number.

^b Determined on a 12.5% polyacrylamide slab gel.

DNA, 80×10^6 , is similar to that previously reported for other NPVs (5, 6, 13).

Although the NPV was present in relatively large amounts in the *G. podocarpi* larvae, it was not necessarily the sole cause of death in those insects. It was possible to purify a small virus from these preparations which was serologically related to the picorna-like virus previously iso-

lated from this species of insect (11, 15). The small viruses (diameter, <35 nm) have been reported to cause inapparent infections in insects, but with a synergistic effect with NPVs they could play a major contribution in insect death. With the commercial use of baculoviruses for insect control, it will be necessary to study in more detail both the effect of inapparent infections in insects and possible viruses contaminating the commercial preparations.

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LITERATURE CITED

1. Aurivillius, C. 1925. Om F. Bryks samlingar i Ost-Afrika. Entomol. Tidskr. 46:206-212.
2. Aurivillius, C. 1930. Lasiocampidae, p. 205-281. In A. Seitz (ed.), The macrolepidoptera of the world, vol. 14. F. Lehmann Verlag, Stuttgart.
3. Austara, O. 1971. *Gonometa podocarpi* Aur. (Lepidoptera:Lasiocampidae). A defoliator of exotic softwoods in East Africa. The biology and life cycle at Muko, Kigezi District in East Africa. East Afr. Agric. For. J. 36:275-289.
4. Brown, D. A., H. M. Bud, and D. C. Kelly. 1977. Biophysical properties of the structural components of

- a Granulosis virus isolated from the cabbage white butterfly (*Pieris brassicae*). Virology **81**:317-327.
5. **Bud, H. M., and D. C. Kelly.** 1977. The DNA contained by NPVs isolated from four *Spodoptera* sp. (Lepidoptera, Noctuidae); genome size and configuration assessed by electron microscopy. J. Gen. Virol. **37**:135-143.
 6. **Burgess, S.** 1977. Molecular weights of Lepidoptera baculovirus DNAs: derivation by electron microscopy. J. Gen. Virol. **37**:501-510.
 7. **Harrap, K. A.** 1970. Cell infection by a nuclear polyhedrosis virus. Virology **42**:311-318.
 8. **Harrap, K. A.** 1972. The structure of nuclear polyhedrosis viruses. I. The inclusion body. Virology **50**:114-123.
 9. **Harrap, K. A.** 1972. The structure of nuclear polyhedrosis virus. II. The virus particle. Virology **50**:124-132.
 10. **Harrap, K. A.** 1972. The structure of nuclear polyhedrosis viruses. III. Virus assembly. Virology **50**:133-139.
 11. **Harrap, K. A., J. F. Longworth, T. W. Tinsley, and K. W. Brown.** 1966. A noninclusion virus of *Gonometa podocarpi* (Lepidoptera:Lasiocampidae). J. Invertebr. Pathol. **8**:270-272.
 12. **Harrap, K. A., C. C. Payne, and J. S. Robertson.** 1977. The properties of three baculoviruses from closely related hosts. Virology **79**:14-31.
 13. **Kelly, D. C.** 1977. The DNA contained by nuclear polyhedrosis viruses isolated from four *Spodoptera* sp. (Lepidoptera, Noctuidae): genome size and homology assessed by DNA reassociation kinetics. Virology **76**:468-471.
 14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
 15. **Longworth, J. F., C. C. Payne, and R. Macleod.** 1973. Studies on a virus isolated from *Gonometa podocarpi* (Lepidoptera:Lasiocampidae). J. Gen. Virol. **18**:119-125.
 16. **Moore, N. F., A. Kearns, and J. S. K. Pullin.** 1980. Characterization of cricket paralysis virus-induced polypeptides in *Drosophila* cells. J. Virol. **33**:1-9.
 17. **Summers, M. D., and D. L. Anderson.** 1973. Characterization of nuclear polyhedrosis virus DNAs. J. Virol. **12**:1336-1346.
 18. **Summers, M. D., and G. E. Smith.** 1978. Baculovirus structural polypeptides. Virology **84**:390-402.
 19. **Wood, H. A.** 1980. Protease degradation of *Autographa californica* nuclear polyhedrosis virus proteins. Virology **103**:392-399.