

The Isolation and Characterization of a Virus from *Oryctes rhinoceros*

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SUMMARY

A virus was isolated from larvae of the rhinoceros beetle (*Oryctes rhinoceros*), which resembled particles observed in thin sections of the midgut epithelium of diseased insects. The virus was rod-shaped, enveloped, and measured approx. 220×120 nm. Purified particles had a density in sucrose of 1.18 g/ml and contained eleven protein components as determined by electrophoresis in 10% polyacrylamide gels. The two major proteins were of low mol. wt. (9.7 and 12.5×10^3). The nucleic acid had a density in caesium chloride characteristic of double-stranded DNA, with an estimated guanosine:cytosine content of 43%. A small proportion (9%) of the DNA was present as covalently-closed molecules. The virus DNA contained molecules with different sedimentation velocities, the major component having a sedimentation coefficient of $57.2S$ and an estimated mol. wt. of 87×10^6 . It is proposed that this virus be included in the *Baculovirus* group.

INTRODUCTION

The rhinoceros beetle *Oryctes rhinoceros* L. (Dynastinae, Scarab., Col.) is an important pest of coconut in the Far East and Pacific islands. The identification of a virus disease affecting this insect led to the development of a biological control programme in which the virus was used, with some success, to control natural populations of *O. rhinoceros* (Huger, 1966; Marschall, 1970). Studies with the electron microscope revealed rod-shaped, virus-like particles in the nuclei of thin sections of infected cells (Huger, 1966; Monsarrat *et al.* 1973a). Recently Monsarrat *et al.* (1973b) extracted and purified a virus from *O. rhinoceros* which, like the particles observed in cells with the electron microscope, was rod-shaped, enveloped, and measured approx. 110×235 nm. The nucleic acid was determined as DNA by colorimetric tests. They suggested that the virus closely resembled *Baculoviruses* (Wildy, 1971), although virus particles were not occluded in crystalline proteinaceous inclusions of the kind observed in nuclear polyhedrosis (NPV) and granulosis virus (GV) infections.

In the present paper, further studies have been made on the nucleic acid and proteins of this virus. The results provide additional evidence that the virus should be included as a member of the *Baculovirus* group.

METHODS

Insect and virus production. Larvae of *Oryctes rhinoceros* were supplied by B. Hurpin (INRA, La Miniere-78, Versailles, France). The larvae were reared individually at 28°C in sealed aluminium containers with a diet of decaying wood and cow manure.

Virus-infected larvae were supplied by K. Marschall (UNDP/FAO Rhinoceros Beetle

Project, Box No. 597, Apia, Western Samoa). A crude virus suspension was prepared by triturating the larvae in distilled water. This suspension was filtered through muslin, and the filtrate administered to second and third instar larvae *per os*. Infected larvae were recognized by the signs of infection described by Huger (1966). Once the larvae became translucent (4 to 28 days after infection), they were removed and stored at -20 °C.

Radioactively-labelled virus was prepared using carrier-free [³²P]-orthophosphate or [³H]-methyl-thymidine (Amersham). Nine days after infection, 10 larvae were injected with 50 µl distilled water, containing 100 µCi [³²P]. Daily injections of the same amount were continued until each larva had received 500 µCi [³²P]. Another group of larvae was injected with a total of 50 µCi [³H]. All larvae were killed by freezing at -20 °C, one day after the final injection.

Electron microscopy. Small pieces of mid-gut tissue removed from second instar *Oryctes rhinoceros* larvae, 17 days after infection, were fixed and embedded as described by Harrap (1972b). Purified virus suspensions were negatively stained with 2% uranyl acetate (Harrap, 1972a). All preparations were examined on an AEI EM 6B electron microscope at a working voltage of 60 kV.

Virus purification. Two preliminary observations were found to be important for virus purification. (1) More virus could be obtained from the gut epithelium than from any other larval tissue. (2) When larvae were triturated in buffers, or distilled water, even at 4 °C, the suspension rapidly became discoloured by the formation of melanin (arising from polyphenol oxidation), which considerably impaired virus purification. The oxidation could, however, be prevented by the use of a reducing agent such as sodium thioglycollate.

Larvae stored at -20 °C were partially thawed, the guts were removed by dissection, and placed in 1% (w/v) sodium thioglycollate at 4 °C. All operations were carried out at 4 °C or over ice. After trituration and filtration through muslin, the filtrate was centrifuged at 2000g for 10 min. The supernatant fluid was then centrifuged at 50000 g for 1 h. The pellet was resuspended in 1% thioglycollate and clarified by low speed sedimentation. The supernatant fluid was layered over 16 ml 10 to 50% (w/v) sucrose gradients prepared in distilled water. The gradients were centrifuged at 45000 g (MSE Superspeed 50, 3 × 20 swinging bucket rotor) for 45 min. A distinct band, containing the rod-shaped, enveloped virus particles was observed two-thirds of the distance down the tube. This component was recovered from the gradient, diluted with distilled water and pelleted by sedimentation at 75000 g for 1 h. The pellet was resuspended in distilled water and clarified by low speed sedimentation.

Such partially-purified virus suspensions were then centrifuged on 25 to 50% (w/w) quasi-equilibrium sucrose gradients at 100000 g for 90 min. The major band (consisting of intact virus particles) in these gradients coincided with a sucrose concentration of approx. 40%, in agreement with results obtained by Monsarrat *et al.* (1973b). This component was removed, diluted, and re-banded on a 30 to 50% (w/w) gradient. The particles present in the 40% sucrose were diluted, and recovered by sedimentation. The resuspended pellet was finally purified on a 10 to 50% (w/v) sucrose gradient.

Purified virus was either resuspended in distilled water, or in 'USM' buffer (8 M-urea, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, in 0.1 M-sodium phosphate, pH 7.8).

Polyacrylamide gel electrophoresis of virus polypeptides. Polyacrylamide gel electrophoresis was carried out by a modification of the method of Lesnaw & Reichmann (1970). Polyacrylamide gels were prepared containing 10% acrylamide, 0.26% N,N'-methylene bis-acrylamide 0.075% ammonium persulphate, 0.06% N,N,N',N'-tetramethylethylenediamine, 0.1% SDS, 8 M-urea, 0.1 M-sodium phosphate, pH 7.8. Gels were pre-run for 30 min at

5 mA per gel in electrode buffer containing 0.1M-phosphate, pH 7.8, 0.1% SDS, 0.1% 2-mercaptoethanol.

Virus samples were disrupted in USM buffer at 100 °C for 1 min. 25 or 50 µl samples were loaded onto each gel, and electrophoresed for 30 min at 2 mA/gel, followed by 5 mA/gel for a further 5 h. The gels were stained in 1% Coomassie brilliant blue in methanol: acetic acid: water (5:1:5), and destained in the same solvent. Gels, stored in 7% acetic acid, were scanned at 550 nm in a Pye-Unicam (Cambridge, England) SP 1800 spectrophotometer.

Estimations of the mol. wt. of the virus proteins were made by the method of Shapiro, Viñuela & Maizel (1967), using 10 µg amounts of the following proteins as standards: transferrin (88000), bovine serum albumin dimer and monomer (136000 and 68000) ovalbumin dimer and monomer (86000 and 43000), lactic dehydrogenase (36000), carbonic anhydrase (29000), α -chymotrypsinogen A (25700), myoglobin (17200) and cytochrome c (11700). All standards were obtained from the Sigma Chemical Co. Ltd.

Analysis of virus DNA. Purified virus, labelled with [^{32}P] or [^3H]-thymidine, was incubated at 37 °C for 1 h in 0.05M-tris-HCl, pH 7.5, 2 mM-MgSO₄ containing 100 µg/ml deoxyribonuclease and 2 µg/ml ribonuclease A, to degrade extraneous DNA or RNA which could be present. After incubation, the virus suspension was layered on a 10 to 50% (w/v) sucrose gradient. Sedimentation and recovery of the virus was as described above.

DNA was extracted from nuclease-treated virus by a lysis procedure similar to that described by Summers & Anderson (1972b). Virus particles were incubated at 60 °C for 20 min in 2% (w/v) sodium N-lauroyl sarcosinate ('sarcosine'), 10 mM-EDTA, 0.004 × SSC (1 × SSC = 0.15M-NaCl + 0.015M-sodium citrate, pH 7.0). Purified, radioactively-labelled, virus particles of the nuclear polyhedroses (NPV's) of *Spodoptera littoralis* and *Spodoptera frugiperda* were also prepared (C. C. Payne, unpublished results) and DNA was liberated by the same method. The NPV particles were not, however, pre-treated with nucleases.

The buoyant density of *Oryctes* virus DNA was measured by comparison with the DNA of *Spodoptera littoralis* NPV, in neutral CsCl gradients formed in an anglehead rotor. (Flamm, Bond & Burr, 1966). A 0.5 ml sample of sarcosine-treated virus was layered over 4 ml 60% (w/w) CsCl in 0.02M-tris-HCl, pH 8.5, in a 10 ml polypropylene centrifuge tube. The tube was filled with liquid paraffin and centrifuged for 60 h at 90000 g and 25 °C. 0.1 ml fractions were collected from the bottom of the tube by displacement of the gradient with liquid paraffin. The density of CsCl at points along the gradient was calculated from the refractive index of a 20 µl sample, measured at 25 °C, using an Abbe refractometer. The labelled DNA was precipitated from the samples at 4 °C after the addition of 80 µg bovine serum albumin, and an equal vol. of 15% (w/v) trichloroacetic acid (TCA). The samples were filtered under gentle suction through Whatman GF 81 glass fibre discs, washed with 10 ml 7.5% TCA and 10 ml absolute ethanol, dried, and counted in a liquid scintillation spectrometer.

CsCl gradients containing ethidium bromide were prepared essentially according to Summers & Anderson (1972b). 1.0 ml of sarcosine-treated virus was layered on 5 ml 54% (w/w) CsCl in 0.01M-tris-HCl, pH 8.0, containing 10 mM-EDTA and 100 µg/ml ethidium bromide. Gradients were formed by centrifuging for 60 h at 60000 g at 25 °C (MSE Superspeed 75, 6 × 15 swinging bucket rotor). 0.2 ml fractions were collected and treated as described above.

Neutral sucrose gradients were prepared 18 to 24 h before use by layering 1 ml samples of 0.125M, 0.25M, 0.5M, 0.75M and 1M-sucrose in 0.1M-sodium chloride, 0.05M-sodium phosphate, pH 6.8 (Burgi & Hershey, 1963). Sarcosine-treated *Oryctes* virus, and NPV

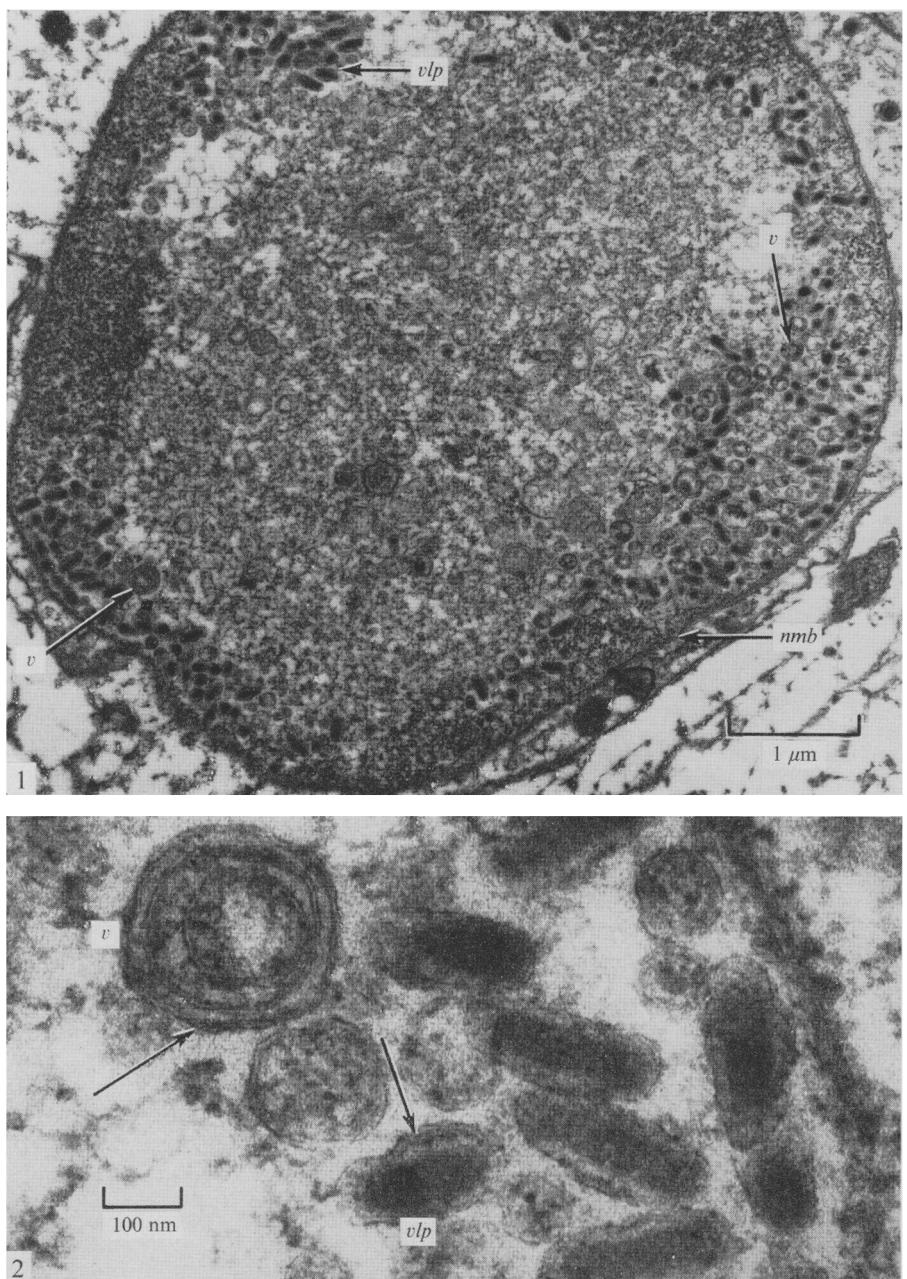


Fig. 1. The virus-infected nucleus of a midgut epithelial cell of *Oryctes rhinoceros*. Virus-like-particles (*vlp*), and many vesicles (*v*) can be seen in a ring-zone adjacent to the nuclear membrane (*nmb*).

Fig. 2. Section showing the double-layered structure (arrowed) of the envelope enclosing the vesicle, and that surrounding the electron-dense core of the *vlp*.

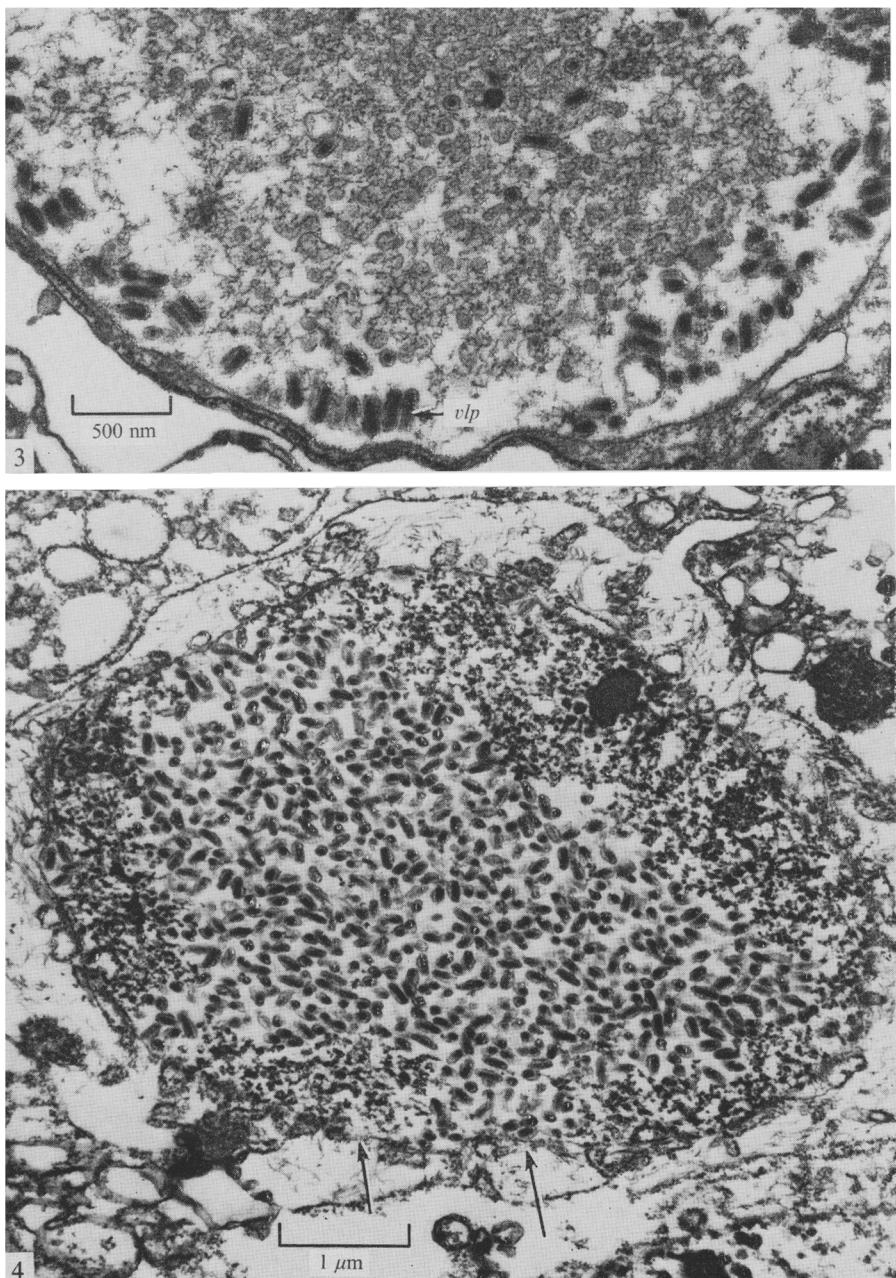


Fig. 3. The apparent alignment of some *vlp*'s at the nuclear membrane.

Fig. 4. An infected nucleus almost filled with *vlp*'s. The nuclear membrane is disrupted (arrowed).

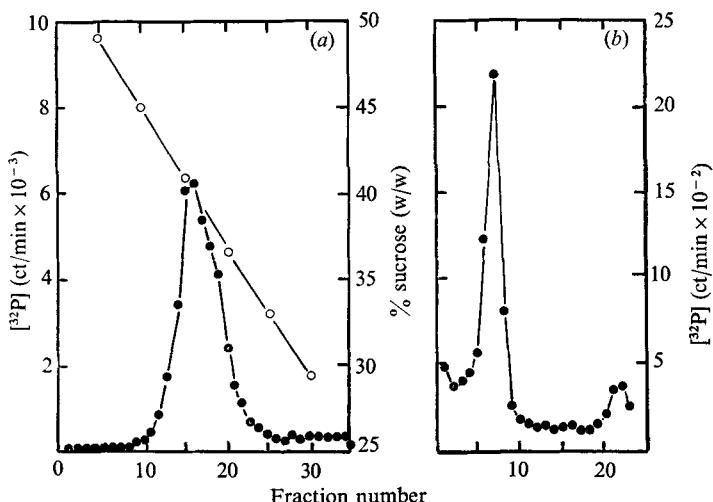


Fig. 5. Sucrose-gradient purification of $[^{32}\text{P}]$ -labelled *Oryctes* virus (a) 30 to 50% (w/w) quasi-equilibrium gradient centrifuged for 90 min at 100 000 g; ●—●, $[^{32}\text{P}]$; ○—○, % sucrose. (b) 10 to 50% (w/v) sedimentation velocity gradient centrifuged for 45 min at 45 000 g.

particles of *Spodoptera frugiperda* were mixed with $[^3\text{H}]$ -thymidine-labelled T4 phage, also treated with sarcosine. 100 μl samples, containing approx. 2 to 4 μg DNA were layered on the gradients and centrifuged at 10 °C for 1 h at 300 000 g (MSE Superspeed 75, 3 \times 6 swinging bucket rotor). The gradients were fractionated as described above.

RESULTS

Electron microscopy of infected tissue

Seventeen days after infection virus-like particles were observed in many nuclei of midgut epithelial cells. Virus morphogenesis occurred within a network of densely-stained material, containing a large number of membrane-bound 'vesicles' (Fig. 1). In section, these 'vesicles' consisted of one or more circular envelopes, each with the apparent structure of a biological unit membrane (Fig. 2). The virus-like particles consisted of a single, densely-stained core, or nucleocapsid, loosely surrounded by an envelope with a structure similar to that of the 'vesicle' membrane. In contrast to the morphogenesis of NPV's (Harrap, 1972b), few naked nucleocapsids were observed. It is possible that formation of the nucleocapsid occurs within the membrane-bound 'vesicles', or that the acquisition of the virus envelope takes place rapidly after the nucleocapsid is formed.

In many infected nuclei, virus-like particles were observed in a ring-zone at the margin of the nucleus (Fig. 1), and in some cases particles were observed apparently aligned at the nuclear membrane (Fig. 3). Fig. 4 probably represents the final stages of infection, when the nucleus is almost completely filled with particles, and the nuclear membrane is showing signs of disruption.

Virus purification

The purification of *Oryctes* virus was monitored using virus particles extracted from $[^{32}\text{P}]$ -labelled larvae. For this study a pure preparation of virus was considered to be one which contained particles of the same density and of the same size. When particles banding

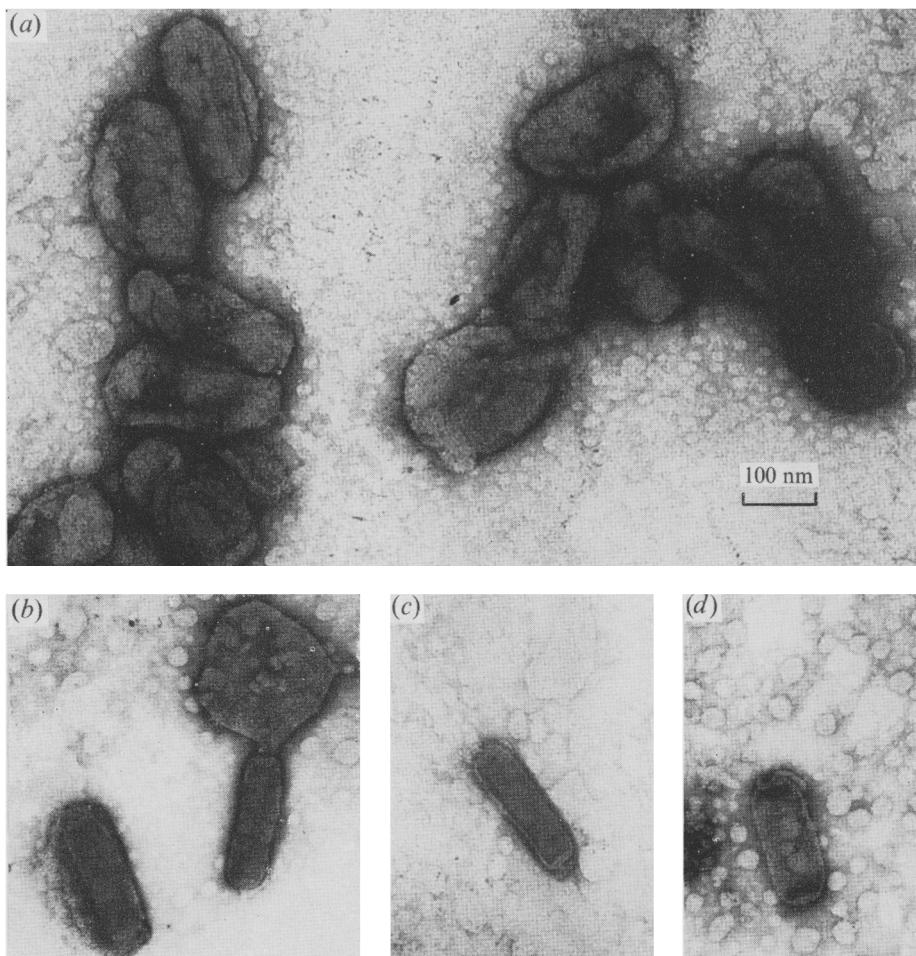


Fig. 6. Negatively-stained preparations of purified *Oryctes* virus. (a) Intact virus particles (b) and (c) nucleocapsids, some of which are still associated with disrupted virus envelopes. (d) An apparently empty nucleocapsid.

at 40% (w/w) sucrose were re-run on a quasi-equilibrium 30 to 50% (w/w) sucrose gradient, a single component was observed (Fig. 5a) with a density in sucrose of approx. 1.18 g/ml. When this component was centrifuged on a sedimentation velocity gradient, most of the [³²P] was present in one band which moved approximately two-thirds of the way down the gradient (Fig. 5b). Virus recovered from this peak therefore satisfied the criteria of one density and one size.

When virus purified in this way was negatively stained and examined in the electron microscope, the majority of the particles were enveloped with typical dimensions of 220 × 120 nm (Fig. 6a). A small number of nucleocapsids (180 × 60 nm) without envelopes, or at various stages of envelope-detachment were also observed (Fig. 6b to d). As described by Monsarrat *et al.* (1973b), the ends of the nucleocapsid appeared thickened or 'capped'.

The infectivity of a purified virus preparation was tested by feeding samples to third instar larvae of *Oryctes rhinoceros*. Six out of ten larvae developed characteristic signs of

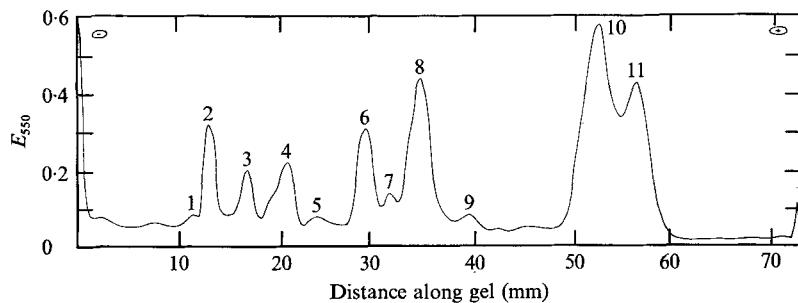


Fig. 7. Polyacrylamide gel electrophoresis of SDS-dissociated proteins of *Oryctes* virus.

Table 1. *The structural polypeptides of Oryctes rhinoceros virus*

Polypeptide	mol. wt. ($\times 10^{-3}$)
I	75.7 ± 1.2*
2	68.5 ± 1.6
3	58.9 ± 1.9
4	48.5 ± 1.7
5	41.3 ± 2.2
6	33.3 ± 1.8
7	29.5 ± 1.4
8	25.3 ± 1.6
9	20.5 ± 0.3
10	12.5 ± 2.1
11	9.7 ± 0.5

* Standard deviation.

the disease within 14 days of feeding the virus, while control larvae showed no signs of infection. It was also possible to isolate virus from the infected larvae, but not from the controls.

Virus proteins

Eleven protein components were resolved by SDS-polyacrylamide gel electrophoresis of disrupted virus in 10% gels (Fig. 7). The mol. wt. of the polypeptides together with standard deviations calculated from eight determinations are shown in Table 1.

Virus nucleic acid

Sarcosine-lysed *Oryctes* virus and *Spodoptera littoralis* NPV were co-run on a neutral CsCl gradient. Peaks of radioactivity (Fig. 8) were observed only within a density range characteristic of double-stranded DNA. The absence of radioactivity at the bottom of the gradient eliminated the possibility that the virus nucleic acid was RNA or single-stranded DNA, as these more dense components would have been pelleted, or banded near the bottom of the tube (Szybalski, 1968).

The DNA released from the NPV and *Oryctes* virus particles did not co-run, the *Spodoptera littoralis* NPV DNA being more dense. Using the slope of the gradient and the relationship between density and guanosine-cytosine (G:C) content (Schildkraut, Marmur & Doty, 1962), it was calculated that the two DNA's differ by 3% G:C, i.e. the *Oryctes* virus DNA has an effective G:C content of 43% compared with a value of 46% calculated for *S. littoralis* NPV DNA (C. C. Payne, unpublished results).

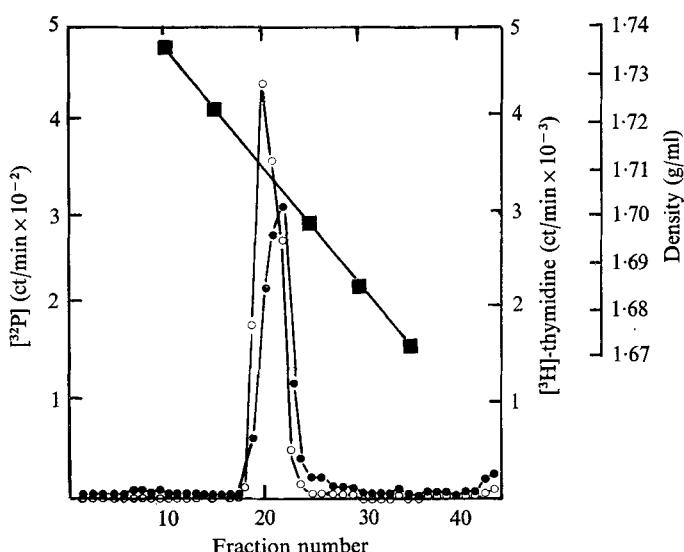


Fig. 8. Neutral CsCl gradient analysis of [³²P]-labelled *Oryctes* virus, treated with sarcosine. ●—●, [³²P]-labelled *Oryctes* virus; ○—○, [^{3H}-thymidine-labelled virus particles of *Spodoptera littoralis* NPV; ■—■, density of CsCl at 25 °C.

CsCl gradients containing the intercalating dye, ethidium bromide, have been used to separate covalently-closed DNA's from open ('nicked' or 'relaxed') circular and linear forms (Radloff, Bauer & Vinograd, 1967). Summers & Anderson (1972a, b, 1973) have shown that a proportion of the DNA from *Baculoviruses* can be isolated in a covalently-closed form.

When sarcosine-treated *Oryctes* virus, labelled with [^{3H}-thymidine, was run on an ethidium bromide-CsCl gradient, two components were observed (Fig. 9). The more dense component, representing approx. 9% of the total radioactivity, was separated from the major peak by a density of 0.04 g/ml. This degree of separation is the same as that between the covalently-closed and open circular or linear DNAs of *Baculoviruses* (Summers & Anderson, 1972a). It therefore appears that a small proportion of the *Oryctes* virus DNA could be isolated as a covalently-closed molecule.

Neutral sucrose gradient analysis of sarcosine-treated *Oryctes* virus is shown in Fig. 10a. The sample used in this analysis did not contain any covalently-closed DNA when run on an ethidium bromide-CsCl gradient. As is commonly found with the DNA of *Baculoviruses* stored for several weeks, any covalently-closed DNA must have been degraded to open-circular or linear forms.

During co-sedimentation with sarcosine-treated T4, the major component of *Oryctes* virus DNA almost co-ran with the marker DNA. Using a sedimentation value of 61.8S, and a mol. wt. of 108×10^6 for T4 DNA (Freifelder, 1970), it was calculated that the major component of the DNA from *Oryctes* virus had a sedimentation constant of 57.2S, and a mol. wt. of 87×10^6 . In addition to this DNA, smaller fractions (33S to 52S) were also observed (Fig. 10a).

In a preparation of *Spodoptera frugiperda* NPV particles which also contained no covalently-closed DNA, two components of 66.1S and 57.9S were detected (Fig. 10b), probably representing open-circular and linear DNA's, respectively. The mol. wt. of the

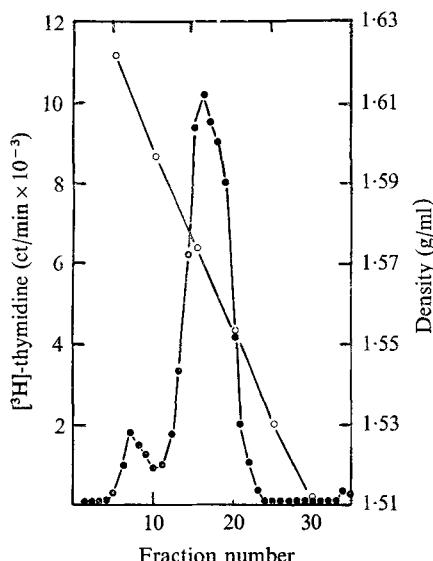


Fig. 9. [^3H]-thymidine-labelled *Oryctes* virus, treated with sarcosine, and centrifuged in CsCl containing 100 $\mu\text{g}/\text{ml}$ ethidium bromide. ●—●, *Oryctes* virus DNA; ○—○, density of CsCl at 25 °C.

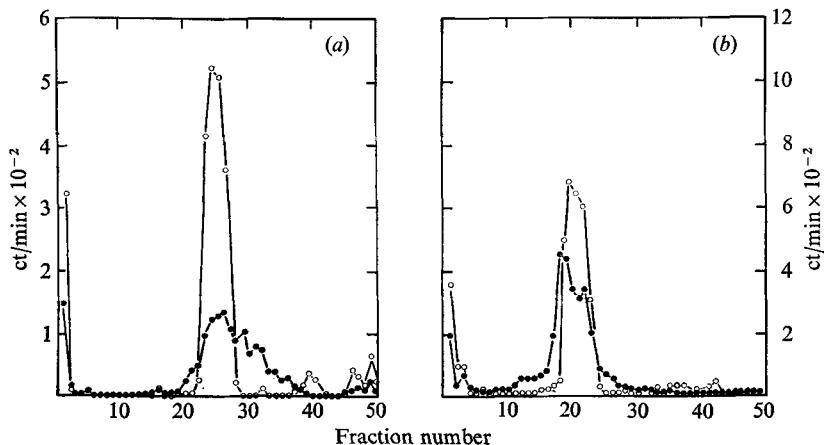


Fig. 10. Rate sedimentation of sarcosine-treated *Oryctes* virus, and virus particles of *Spodoptera frugiperda* NPV, on neutral sucrose gradients. (a) ●—●, *Oryctes* virus DNA labelled with [^{32}P]; ○—○, T4 DNA labelled with [^3H]-thymidine. (b) ●—●, *Spodoptera frugiperda* NPV DNA labelled with [^{32}P]; ○—○, T4 DNA labelled with [^3H]-thymidine.

slower-moving component was 90×10^6 , in good agreement with the size of the major fraction of *Oryctes* virus DNA, and with previously published values for linear forms of *Baculovirus* DNA's (Summers & Anderson, 1972b, 1973).

DISCUSSION

Details of *Oryctes* virus morphogenesis, observed in the nuclei of midgut epithelial cells, were similar to those described by Huger (1966) and Monsarrat *et al.* (1973a). They also

closely resemble the development of a virus-like particle in midgut cells of *Gyrinus natator* L. (Gouranton, 1972). The absence of paracrystalline inclusions containing virus particles, and the proliferation of membranous material distinguish this morphogenesis from that observed in most *Baculovirus*-infected cells (Harrap, 1972*b*), although in both NPV and GV infections of the gut epithelial cells of Lepidoptera, virus particles are not generally found in proteinaceous inclusions (Harrap & Robertson, 1968).

The nucleocapsids and enveloped virus particles which were present in purified preparations of *Oryctes* virus were structurally similar to *Baculovirus* particles, although measurements indicated that particles of *Oryctes* virus are generally wider and shorter than baculoviruses in which a single nucleocapsid is enveloped within a membrane (Harrap, 1972*a*). The characteristic 'capping' or thickening of the ends of the nucleocapsid can also be seen in some micrographs of NPV particles (Harrap, 1972*a*).

Eleven protein components were resolved in purified preparations of *Oryctes* virus. The major proportion of the virus is composed of the two smallest polypeptides, which may constitute as much as 50% of the total protein. A direct comparison with the proteins of *Spodoptera frugiperda* NPV particles suggests similarities in the general profile, although the electrophoretic mobilities, and hence the mol. wt. of individual polypeptides are not strictly comparable (C. C. Payne, unpublished results). Antigenic similarities between *Baculoviruses* and *Oryctes* virus have been suggested by Monsarrat *et al.* (1973*a*), when using antisera prepared to the granulosis virus of *Mamestra oleracea* L. to examine antigens in *Oryctes* virus-infected cells. We have also detected cross-reactions in gel diffusion tests between *Oryctes* virus and antisera prepared to NPV particles (K. A. Harrap & C. C. Payne, unpublished results) and this aspect of the work is at present under further investigation.

The density of the nucleic acid of *Oryctes* virus in caesium chloride confirms that it is DNA and probably double-stranded. As with *Baculoviruses* (Summers & Anderson, 1972*a, b*, 1973), a small proportion of the DNA can be recovered in a covalently-closed form. The relatively large amount of open-circular or linear DNA extracted from the virus may have resulted from nuclease activity during purification. The major DNA fraction in neutral sucrose gradients was comparable in size to the linear DNA forms of several *Baculoviruses* including *Spodoptera frugiperda* NPV (Summers & Anderson, 1973). The presence of fragments with lower sedimentation values indicated that the DNA preparation was heterogeneous in size. The use of deoxyribonuclease in the purification of virus particles may also have accounted for this.

The properties of *Oryctes* virus reported here and by Monsarrat *et al.* (1973*a, b*) strengthen the view that the virus should be included within the *Baculovirus* taxonomic group. The previous use of the term '*Rhabdionvirus Oryctes*' (Huger, 1966) to identify the virus, is now misleading as it implies a possible association with the *Rhabdovirus* taxon, and it is suggested that this name should be discontinued.

After the experimental study of *Oryctes* virus was completed, and during the preparation of this paper, my attention was drawn to a recent publication by Revet & Monsarrat (1974) in which the structure of the nucleic acid of *Oryctes* virus was examined in detail. The results presented above confirm the findings of these workers.

I would like to thank Dr K. A. Harrap for the electron micrographs and other members of the Unit of Invertebrate Virology for their expert assistance.

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