Isolation and Structure of a Nuclear Polyhedrosis Virus from Polyhedra of the Silkworm, *Bombyx mori*

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Rate-zonal centrifugation of virus particles released from the alkaline solution of inclusion bodies from the silkworm larva showed that the nuclear polyhedra contained at least three different forms of the virus particles. These forms of the virus were separated in sucrose density gradient centrifugation. The best result of separation was obtained when Tris-buffer was used as gradient buffer. The nuclear polyhedra in the silkworm larva contained 80% of single and 20% of bundle virus rods. All of the separated virus had a developmental membrane. Detail studies on the relative infectivity of both the single and the bundle virus rods showed that they were almost equally infectious in hemocoelic inoculation to the silkworm pupa. The naked virus rod, i.e., virus particle having only an intimate membrane did not occur significantly under the present condition of investigation.

INTRODUCTION

The nuclear polyhedrosis virus of the silkworm was the first insect virus to be demonstrated by the electron microscope (BERGOLD, 1947). In this virus, there is usually only one rod enclosed in a developmental membrane, but occasionally there are two and very rarely three rods within one developmental membrane (BERGOLD, 1958). Four to 18 rods within a developmental membrane, however, have been reported in the silk gland cell of the silkworm (ARUGA, et al., 1963). The highly infectious particles of a nuclear polyhedrosis virus of the silkworm have a developmental membrane (BERGOLD, 1958). The morphology of the nuclear polyhedrosis virus of the silkworm liberated from the inclusion body has been reported by several investigators (BERGOLD, 1958, 1963; KHOSAKA et al., 1971; KOZLOV and ALEXEENKO, 1967; KRIEG, 1961). However, the virus composition of an inclusion body and the infectivity of the different virus forms, such as virus bundles, have not been established. In the present study, the virus composition of the silkworm nuclear polyhedra and the infectivity of the different virus forms were investigated employing sucrose density gradient centrifugation.

MATERIALS AND METHODS

Preparation of polyhedra: Infected larval hemolymph was filtered through 4 layers of cheese cloth, and the filtrate was immediately centrifuged at 1,000 *g* for 20 min. The supernatant was removed by suction and the sedimented polyhedra were suspended

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in 25% (w/v) sucrose dissolved in water. The suspension was centrifuged 1,000 *g* for 20 min and the sedimented polyhedra were suspended in 25% sucrose solution. The suspended polyhedra in a volume of 10 ml was layered on 30 ml of 50% sucrose solution and the polyhedra were sedimented through 50% sucrose at 1,800 *g* for 40 min in a 50 ml centrifuge tube. The differential centrifugation was repeated twice and the polyhedra were washed several times with de-ionized distilled water. The polyhedra were finally dried *in vacuo* and stored at -20°C , until use.

Liberation of virus particle: The virus particles were liberated from the polyhedra with 1 ml of 0.08 M Na_2CO_3 + 0.025 M NaCl per 10 mg of dried polyhedra. The reaction mixture was incubated at 27°C for 30 min in a water bath with gentle stirring every 5 min. Then the reaction mixture was centrifuged at 2,000 *g* for 20 min and the impurities were sedimented. The liquid supernatant was layered on a sucrose gradient.

Density gradient centrifugation: Usually 2 ml of virus sample were layered on a 28 ml of linear sucrose gradient (20—40%, w/v) in 0.03 M Tris-HCl buffer, pH 7.6 (Tris) or in 0.05 M potassium phosphate buffer, pH 7.2 (Phos). The virus zone banded at approximately the center of the tube after centrifugation for 40 min at 5°C and 41,400 *g* in Spinco SW 25.1 rotor on Spinco L₅-50 ultracentrifuge. The fractions were collected from the bottom of the tube with Spinco fraction recovery system and were assayed for absorbancy at 260 nm in a Beckman DU-2 spectrophotometer.

Electron microscopy: Electron micrographs were taken employing a JEM-7A EM operating at 80 KV and at a magnification of 50,000. Samples were fixed with 3% glutaraldehyde in Phos-buffer, for overnight at 5°C and dialyzed for 24 hr against Tris-buffer. Negatively stained samples were prepared with 2% uranylacetate, pH 4.6.

Biological assay: The virus suspension was diluted decimally with Phos. Of each dilution 0.005 ml were inoculated into the hemocoel of a silkworm pupa (Strain: Shûkô × Ryûhaku). The pupa were reared at 28°C for 10 days and then examined for the presence of nuclear polyhedra with a light microscope. Median lethal dose (LD50) was calculated according to the method of REED and MUENCH (1938).

RESULTS

Separation of virus particles

The alkali liberated virus suspension was layered on the Tris-buffered sucrose gradient and centrifuged. After the run a main virus band occurred in the center of the gradient column (Fig. 1). Also, two small bands were formed below the main band. These three virus bands were designated as band I, II, and III from the center to the bottom of the tube. The virus band I comprised single rod virus with a developmental membrane (Fig. 2). Other bands (II and III) were virus bundles within a developmental membrane. Band II consisted of two rods within a developmental membrane (Fig. 3). Three virus rods within a developmental membrane were found in the case of band III.

Separation of the virus particles in the phosphate-buffered sucrose gradient was not satisfactory, although three virus bands were formed. Many single rods were observed to be present in all 3 virus bands. The virus band having naked rods, i.e., virus rods having only intimate membrane, was not observed significantly in both the Tris- and phosphate-buffered sucrose gradients.

Fig. 1. Sedimentation profile of a nuclear polyhedrosis virus of the silkworm liberated from the polyhedra.

Fig. 2. Purified single rod virus of the silkworm nuclear polyhedrosis virus from band I negatively stained with 2% uranylacetate. $\times 66,000$. A single rod within a developmental membrane,

Fig. 3. Double rod virus from band II separated in the Tris-buffered gradient. All virus rods with developmental membranes. $\times 66,000$.

Negative staining of the virus

To avoid the disruption of the developmental membrane during negative staining, the separated virus particles were fixed by glutaraldehyde before staining. Several experiments using phosphotungstic acid (PTA) demonstrated that PTA badly damaged the developmental membrane, especially when the samples were not fixed. However, negative staining by uranylacetate did not affect the membrane structure of the virus. Combination of prefixation and staining with uranylacetate were most effective for the preservation of the intact form of the virus.

Recovery of the virus

Recovery of the silkworm nuclear polyhedrosis virus in the sucrose gradient was studied (Table 1). More than 80% of the infectivity were recovered in the virus bands. The single rods occupied about 80% of the infectivity which were recovered in the sucrose gradient. The bundle virus separated in band II and III were not more than 20% of total infectivity in bands. The high yields of the virus indicated that there were very little inactivation during separation. The silkworm nuclear polyhedra in the larva were typical single-rod virus polyhedra (MARTIGNONI et al., 1971).

Table 1. RECOVERY OF THE SILKWORM NUCLEAR POLYHEDROSIS VIRUS SEPARATED IN THE Tris-Buffered SUCROSE DENSITY GRADIENT

		Exp. 1		Exp. 2		Exp. 3	
		Infectivity ^b (LD 50/ml)	%	Infectivity (LD 50/ml)	%	Infectivity (LD 50/ml)	%
Total infectivity	applied ^a	$10^{8.1}$	100	$10^{7.7}$	100	$10^{8.0}$	100
Infectivity recovered in virus bands	Band I	$10^{8.0}$	80	$10^{7.6}$	80	$10^{8.0}$	80
	Band II	$10^{6.9}$	6.3	$10^{6.8}$	13	$10^{6.9}$	6.3
	Band III	$10^{5.1}$	0.10	$10^{5.1}$	0.25	$10^{5.3}$	0.16
	Total	1.1×10^8	86	4.7×10^7	93	1.1×10^8	87

^a The virus was liberated from 20mg of polyhedra.

^b Virus titer in LD 50 (hemocoelic inoculation; dose: 0.005 ml/pupa).

Table 2. COMPARATIVE INFECTIVITY ASSAY WITH *Bombyx mori* ALKALI LIBERATED NUCLEAR POLYHEDROSIS VIRUS IN THE Tris-Buffered SUCROSE GRADIENT

Virus band	Amount of virus (ODU at 260 nm ^a)	Infectivity ^b (LD 50/ml)	Relative infectivity (LD 50/ODU at 260 nm)
Ia	1.97	10 ^{7.7}	10 ^{7.4}
Ib	1.20	10 ^{7.9}	10 ^{7.8}
Ic	0.570	10 ^{6.8}	10 ^{7.1}
IIa	0.240	10 ^{6.5}	10 ^{7.1}
IIb	0.135	10 ^{6.9}	10 ^{7.8}
IIIa	0.090	10 ^{4.8}	10 ^{5.8}
IIIb	0.065	10 ^{4.7}	10 ^{5.9}

^a Optical density unit: 1 ODU is the amount of material having a optical density of 1 with a light pass of 1 cm (JOKLIK, 1962).

^b Virus titer in LD 50 (hemocoelic inoculation; dose: 0.005 ml/pupa).

Comparative infectivity assay of the virus particles

To assay the comparative infectivity associated with each of the virus forms in bands, the tubes containing maximum amounts of the virus in each band were taken and assayed. Differences of relative infectivity (LD50/optical density unit at 260 nm) between the single rod virus and the bundle virus were compared. Results are shown in Table 2. There were no significant differences in relative infectivity between the single rod and the double rod virus. The virus particle which contained three rods in a developmental membrane was also infectious, but its infectivity was slightly lower than that of the single rod virus.

DISCUSSION

Since BERGOLD (1958) stressed the need for standard condition for the liberation, with alkali, of the virus particles occluded in the inclusion body of insect viruses, several workers have standardized their methods by dissolving the inclusion bodies in weak alkali solutions for 2 to 3 hr under cold conditions (KHOSAKA et al., 1971; KOZLOV and ALEXEENKO, 1967; SUMMERS and PASCHKE, 1970). We have employed, however, strong alkali and high temperature conditions for the liberation of virus particles occluded in the polyhedra. This resulted in the satisfactory recovery, from polyhedra, of the liberated virus particles which were infectious. No bad effects on the infectivity of the virus particles have been detected.

Rate-zonal centrifugation of the virus particles liberated from the silkworm polyhedra demonstrated three sedimenting virus particle forms. All the virus forms separated in the sucrose gradient had a developmental membrane. Occurrence of naked rods in the sucrose gradients have also been reported (KHOSAKA et al., 1971; KAWANISHI and PASCHKE, 1971; SUMMERS and PASCHKE, 1970). We have not found naked rods significantly in our preparation.

The effects of buffer pH and ionic strength on the yield of virus particles obtained by dissolving *Rachiplusia ou* nuclear polyhedra were investigated (KAWANISHI and PASCHKE, 1971). However, the effects of sucrose gradient buffer on the sedimentation profile and separation of the virus particles were also important in the purification

of the silkworm nuclear polyhedrosis virus by sucrose density gradient centrifugation. Phosphate-buffered gradients have been used for the purification and the characterization of nuclear polyhedrosis viruses of Lepidopterous insects (KAWANISHI and PASCHKE, 1971; KHOSAKA et al., 1971; HARRAP, 1972). In the Tris-buffered sucrose gradient, the silkworm nuclear polyhedrosis virus sedimented according to the number of rods in a developmental membrane. In the case of the phosphate-buffered gradient, the sedimentation profile was different and the amount of the lower virus bands increased, but the peak of single rod was markedly reduced without loss of total virus recovery. However, no differences could be seen electron-microscopically between the virus particles of band I and those of lower virus-bands. Possibly, the single rod virus loosely aggregated and banded in the lower virus bands in the phosphate-buffered sucrose gradient.

BERGOLD (1958) reported that the virus particles having developmental membrane were the highly infectious forms of the nuclear polyhedrosis viruses. Recently, KHOSAKA and HIMENO (1972) reported that a naked rod and rod within a developmental membrane were almost equally infectious to the silkworm pupa by hemocoelic inoculation. When the virus forms separated by the sucrose gradient were compared on their infectivity, no significant differences were found between the single rod virus and the double rod virus. However, the naked rod which has occurred naturally in the infected hemolymph of the silkworm was more infectious than the rod within a developmental membrane (KAWARABATA, unpublished data). Further studies on the highly infectious form of the nuclear polyhedrosis virus will be presented in another paper.

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