PECULIAR AND IMPORTANT CONTRIBUTIONS OF CYTOPLASMIC POLYHEDROSIS VIRUSES (CPV) IN BIOTECHNOLOGY, VIROLOGY, PHYSIOLOGY, AND BIOLOGICAL CONTROL OF INSECTS

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1. INTRODUCTION

Insect viruses are widely studied both as biological control agents of insects and expression vector of foreign genes. Among theses viruses, the baculoviruses received the most attention mainly because of their lethal effect on the insect population as well as their successful replication and production in insect tissue culture systems. However, along with the baculoviruses, another group of occluded viruses such as the cytoplasmic polyhedrosis viruses (CVP) present important charasteristics. Historically, CPV's were first studied as noxious microorganisms and contaminants of beneficial insect colonies. Observation of abnormal presence of dense inclusion bodies in the midgut of the silkworm *Bombyx mori* leaded Japanese scientist to the first diagnosis, in 1938, of Cytoplasmic Polyhedrosis Viruses. Later, it was demonstrated that many other economic important insects collected in nature or reared in the laboratory, could be infected par different CPV's. The preventions of CPV infections were therefore the main initial research objectives. However, later on, important fundamental data in virology and in molecular and cell biology, such as the discovery of the CAP structure of messenger RNA were obtained from research on *Bm* CPV. Moreover, the numerous physiological and metabolic disturbances produced as a consequence of CPV infection can act as important potentiators and enhancers of insect mortalities. This, combined with the persistence of viral infection throughout insect generations can be exploited in the development of biopesticides. In this paper, we will discuss on the characteristics of the cytoplasmic polyhedrosis viruses which merit to be exploited in important fundamental as well as applied researches.

Classification, physical and molecular characteristics of the CPVs

The cytoplasmic polyhedrosis viruses (Fig.1) are segmented double-stranded RNA viruses and members of Reoviridae. According to the electrophoretic pattern of the ten viral dsRNA segments on a 3% polyacrylamide gel, 12 types of CPV's were originally classified [1, 2]. Later on, two types of CPV's were newly identified [3, 4], and analysis of RNA of a CPV from the spruce budworm, *Choristoneura fumiferana* [5] showed the presence of a new additional CPV. From twenty years ago to very recently the 10 viral RNA segments were cloned and sequenced and their interesting functions were analyzed. An insect can be infected with different types of CPV's (Fig.2) which leads to resolution on gel of more than 10 ds RNA segments.

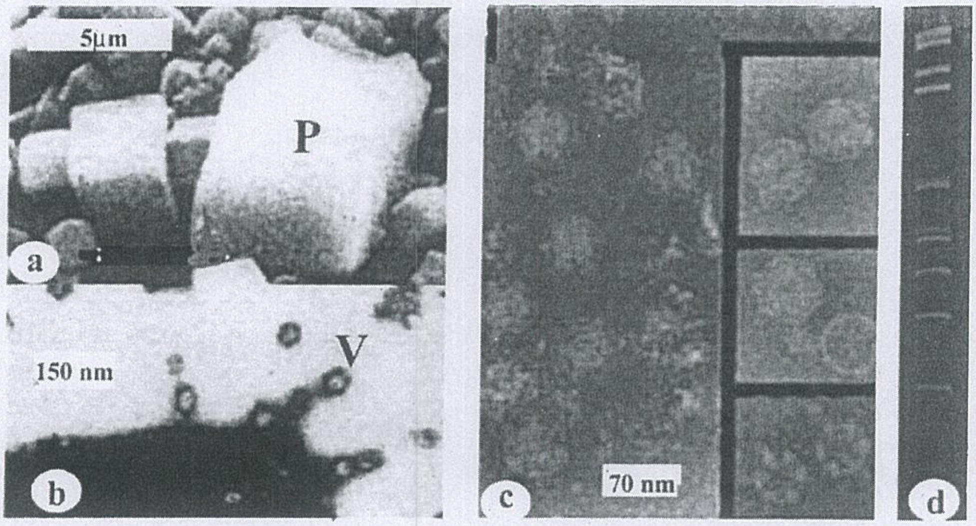


Figure 1a CPV polyhedra (P). Scanning electron microscopy.
Figure 1b Virions (V) released by alcaline dissolution of polyhedra as in an insect midgut.
Figyre 1c Icosahedral CPV virus particles.
Figure 1d Electrophoretic pattern of double-stranded RNA of CPV

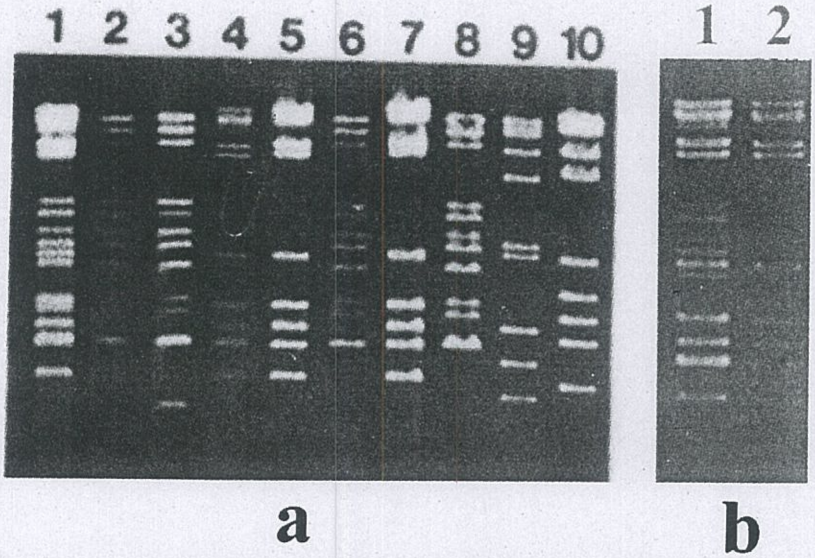


Figure 2a Resolution of more than 10 ds RNA segments on agarose gel from infected *H. armigera* midguts (1-10): Mixed CPV types infection.
Figure 2b Separation of one Ha CPV type (2) from the mixture 1 by passage on cell line

The polyhedra consist of many virus particles and a crystalline matrix protein (polyhedrin). One of the functions of the polyhedrin, massively synthesized late during infection is to protect virions from physicochemical inactivation in the environment conditions. The polyhedrin has a molecular weight ranging from 27,000 to 31,000. An alkaline protease is associated with polyhedra derived from infected larvae but not with those obtained from infected cell cultures. The alkaline protease might activate the dissolution of polyhedra in the midgut. The size and shape (cubic, icosahedral...) of the polyhedron is variable according to virus strain and insect cell.

Occludded or non-occludded virions are identical. The icosahedral virus particles has a diameter of 55-69 nm and one hollow spike located on each of the twelve vertices of the shell. Absence of a double-shelled structure differentiate the capsid of CPV from other *Reoviridae*. The icosahedral viruses have twelve spikes, one at every vertex of the icosahedron.

Viral replication in insects and cells cultured *in vitro*

When ingested by larvae, the CPV polyhedra are dissolved in the midgut by alkaline insect juice. In this way, the released virions are extracted from the protective polyhedra and attach to the midgut cells. The first interaction step is not well documented. The new virus particles are noted 3 hours later in the cytoplasm and the titer increases for the next 24 hours. High amounts of viral particles are detected in the larval hemolymph [6, 7], but intracellular virus particles are noted only in the midgut cells. In viroplasm structures, localized in infected cells, RNA and protein matrices, capsids, core and virions are found. First signs of crystallization of polyhedrin as polyhedron are noted approximately 15 hours post infection. At the end of the viral infection, the cell cytoplasm is filled with polyhedra that contain from one to more than a thousand virions. The midgut is enlarged and acquires a milky white color (Fig. 3). Later on, the infected cells desquamate and are released intact or ruptured in the feces. The viral polyhedra spoiling other plants and soil contribute to the horizontal propagation of the viral infection. Before the desquamation process, some non-occluded virions pass into newly generated differentiated midgut cells through the plasma membrane. These cells will support in their turn the entire CPV replication cycle. By this mechanism, the viral infection persists for a longer period in the insect and therefore the larva acts as a virus amplification factory (Fig. 4).

Non-occluded virions in crude extracts of infected midgut as well as those extracted following *in vitro* alkaline dissolution of the polyhedra are equally infectious for insect cell cultures. Several insect cell lines are able to support the replication of CPV's (Fig. 5). The general pattern of the viral morphogenesis in *vitro* (Fig. 6) is similar to the one observed and described earlier in the larvae. Polyhedra are noted 15 hours post infection [8] and later on numerous polyhedra containing virions fill the cell cytoplasm. Cubical shape characterizes most of the CPV polyhedra at the end of an *in vitro* infection. However, for some CPV strains other crystallization patterns could be observed. It is now assumed that interactions between the viral genome and the cell culture conditions will influence the crystal formation and the shape of the polyhedra. Spherical and cubical polyhedra that accounted for 10% and 90% of the total polyhedra, respectively, were observed in EsCPV-infected midgut *of E. scandens* larvae [8]. After the passage in Lymantria dispar and several other cell cultures [8, 9], only cubical polyhedra were formed. When *E. scandens* larvae were fed with cubical polyhedra obtained from cell culture, the two types of polyhedra reappeared. These data could indicate that some cell factors or conditions

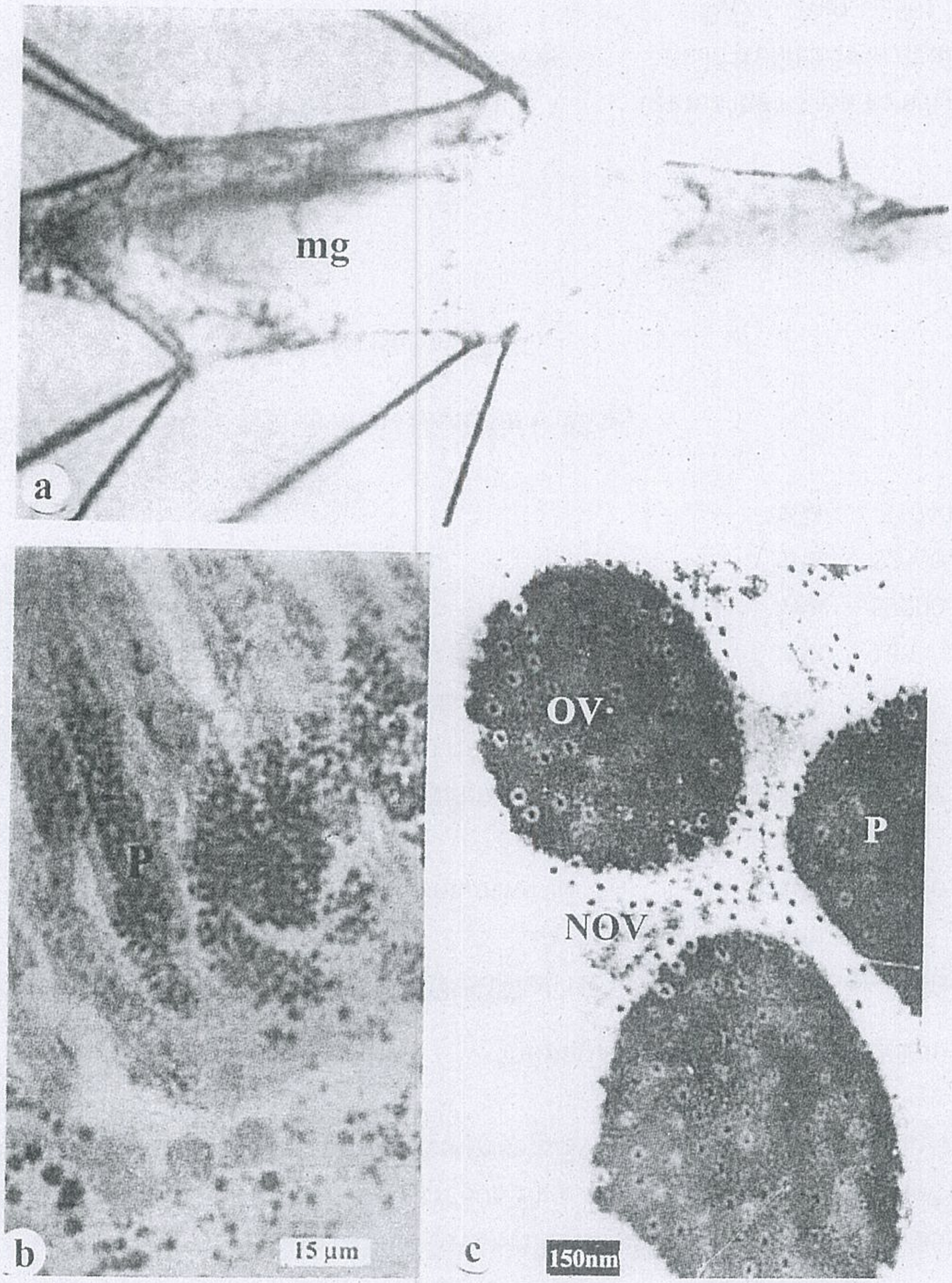


Figure 3a Milky white color of CPV infected midgut (mg)
Figure 3b CPV polyhedra (P) in epithelial midgut infected cells Light microscopy.
Figure 3c CPV polyhedra (P) in epithelial midgut infected cells Electron microscopy.
Non-occluded (NOV) and occluded (OV) virions.

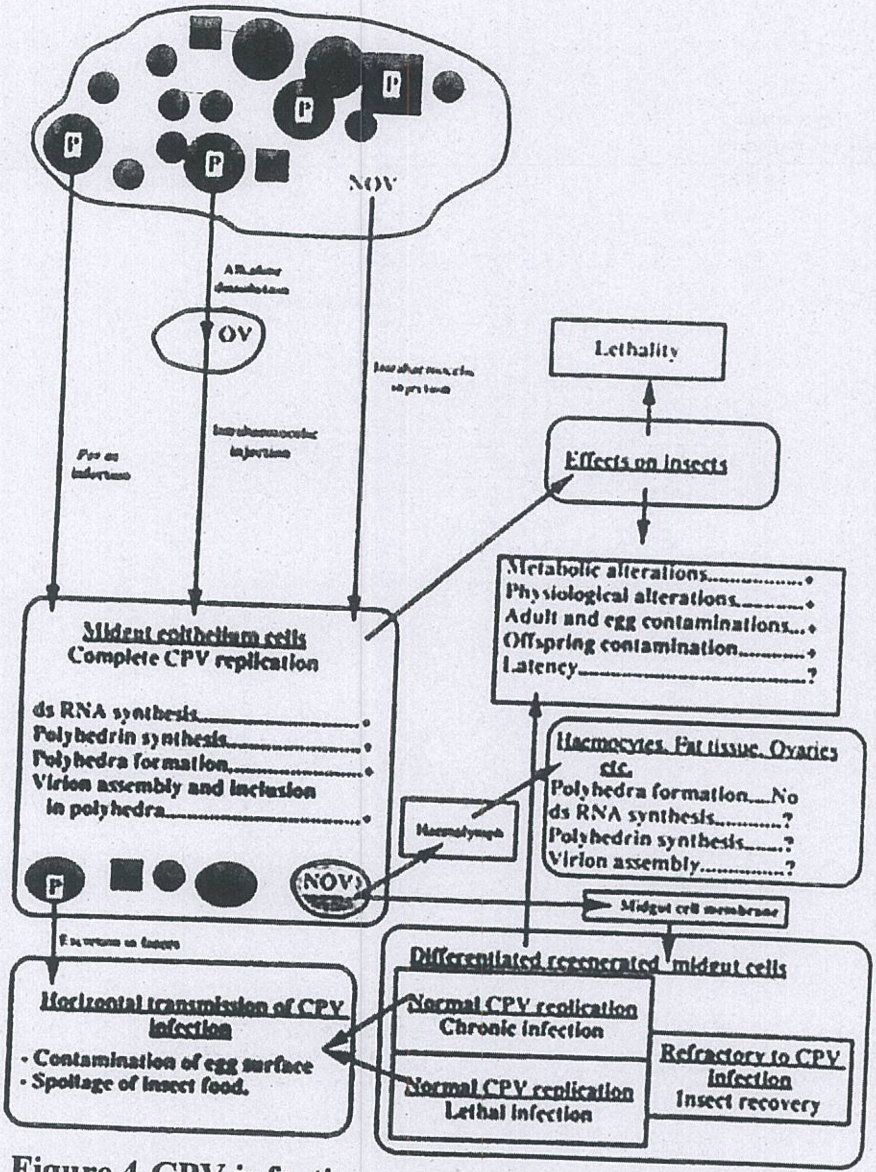


Figure 4 CPV infection patterns in the insect.
NOV: Non-occluded virions OV: Occluded virions
P: Polyhedra
From S. Belloncik, 1996.

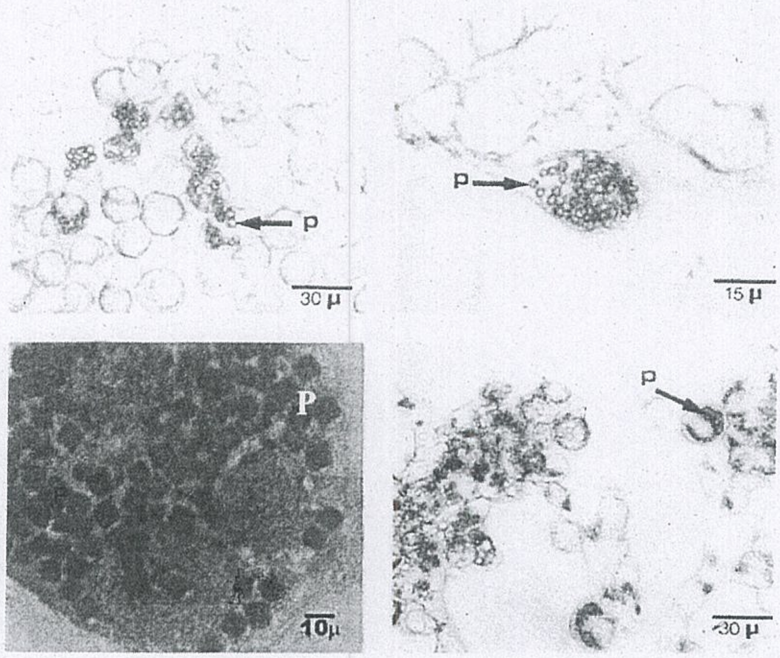


Figure 5 Susceptibilty of different cell lines to cytoplasmic polyhedrosis viruses. P: Polyhedra.

present in insect cells are necessary in the process of polyhedrin crystallization and polyhedra maturation.

Different biosynthetic polyhedrins obtained in several laboratories were used to study some crystallization pattern of CPV polyhedrin. Cloned cDNA encoding the polyhedrin of BmCPV was introduced into an expression vector and expressed in *Escherichia coli* [10]. A polypeptide of the same molecular weight as natural polyhedrin was synthesized that reacted with polyhedrin antiserum. The expressed polyhedrin did not form any crystalline structure in *E. coli* but instead accumulated in the form of an insoluble inclusion body. Later, expression of EsCPV polyhedrin gene in BmN4 cells using a Bm baculovirus expression vector led to the formation of cubical nuclear CPV polyhedra [11]. When the same gene was expressed in the BoMo silkworm cell line as well as in silkworm larvae using a BmNPV polyhedrin expression vector, nuclear and cytoplasmic CPV polyhedra were formed [12]. The use of P10 expression vector further demonstrated the concomitant expression and crystallization of NPV and CPV polyhedrin in a same insect larval cell and in cells cultivated in Fig. 7 [12]. Studies on recombinant polyhedrin of

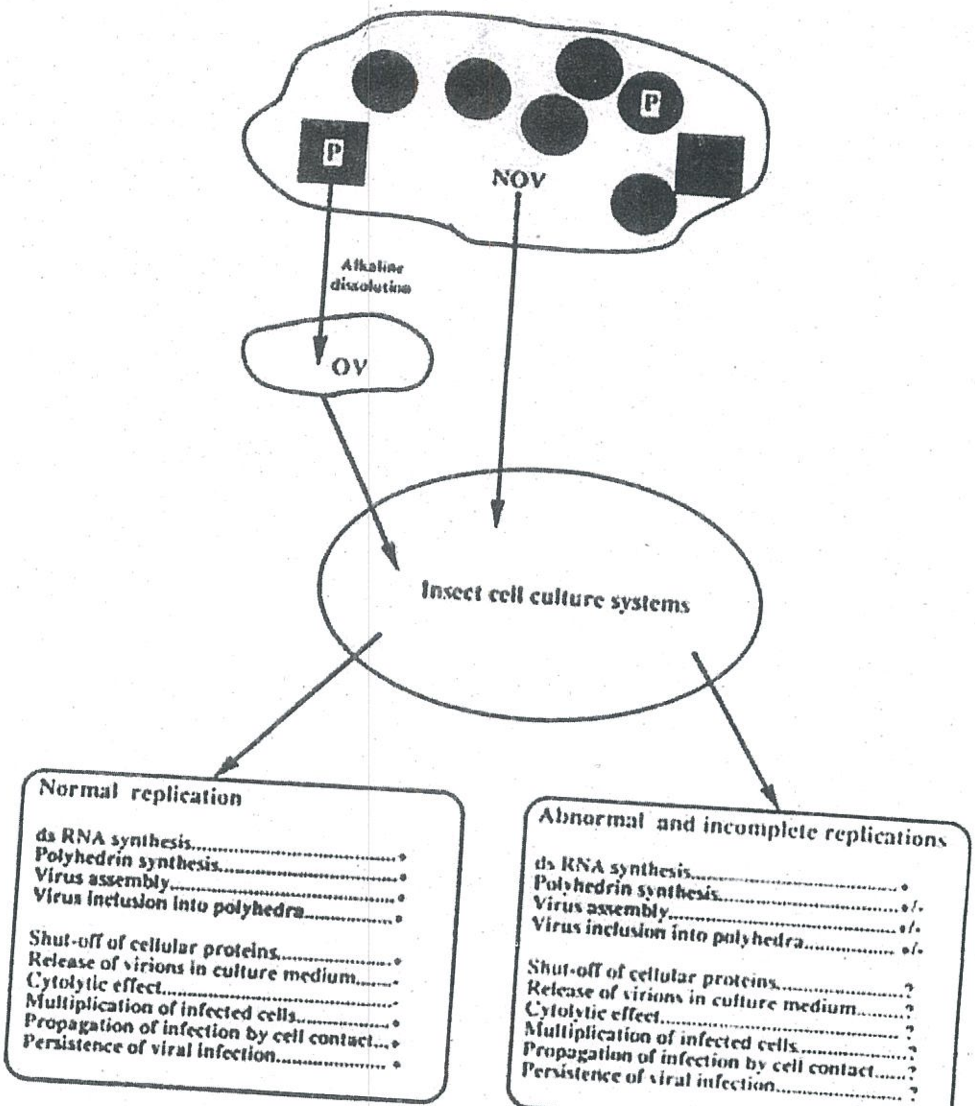


Fig 6 **Replication patterns of CPV's in a cell culture system**
NOV:Non-occluded virions. OV: occluded virions
P: Polyhedra
From S. Belloncik, 1996.

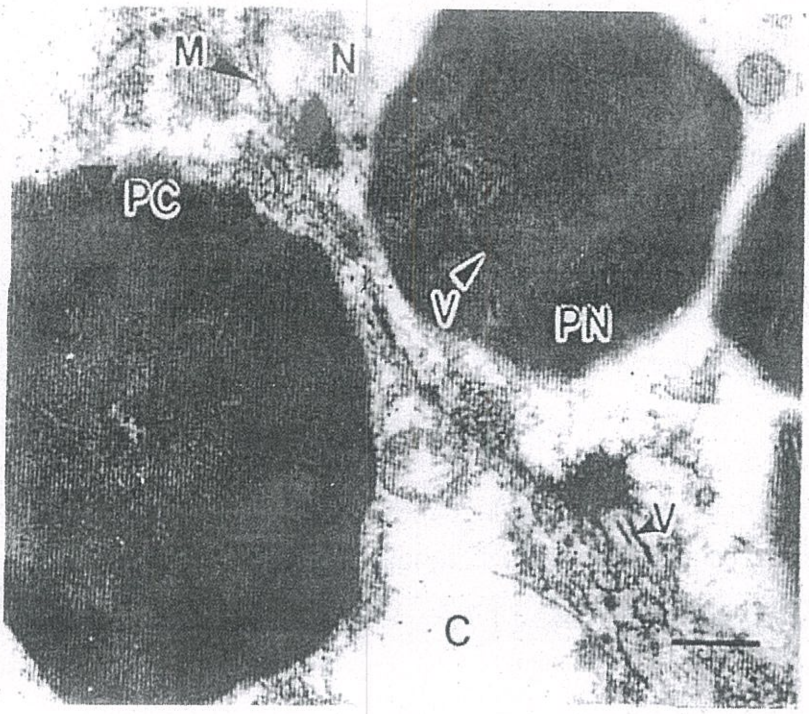


Figure 7 Co-crystallization of recombinant CPV (PC) and native NPV (PN) polyhedrins V: baculovirions.

different natural and mutant *B mori* CPV"s demontrated that the shape of polyhedra as well as the crystallization pattern of the polyhedrin could be changed by mutations as respectively N-terminal and C-terminal regions of the polyhedrin gene [13].

The difference in viral susceptibility permitted the separation of CPV strains from a mixed CPV isolate (Fig. 2) [4]. Besides cell susceptibility to viral infection, some other factors promoting CPV replication could be present. High titers of virions are detected in the insect hemolymph but no polyhedra are observed in the hemocytes from infected insects. However normal CPV morphogenesis occurs in these cells if they are transferred to an *in vitro* culture [7].

No lysis of infected cells is normally associated with CPV infection in vitro [4, 8]. CPV infected cell cultures can normally be maintained and passaged for several months or years without cell destruction (Fig. 5), and no shut-off of cell protein synthesis is noted. The viral infection spreads in a cell culture monolayer [4]. No CPV particles are released from the cells into the culture medium. The viral propagation was demonstrated to be due to the multiplication of infected cells [4] as well as the passage of non-occluded virions from cell to cell through plasma membranes (Belloncik, unpublished results).

Physiological perturbations, viral latency and persistence in insects

Studies conducted in our laboratory as well as those described by Payne [14] demonstrated that the lethality of CPV could depend on the CPV strain (Tables 1 and 2). Some CPV's are highly lethal while some others are not. It is important to point out that a CPV strain could not be lethal in laboratory conditions but kill insect larvae very efficiently when applied in nature [15]. In contrast with other insect viruses such as baculoviruses, the general pattern of a CPV infection in an insect population is a slow, less lethal, chronic and more persistent infection. A high proportion of heavily-infected larvae can reach the pupal and adult stages. However, the destruction of the CPV infected midgut of these larvae leads to nutritional deficiency and indirectly to severe metabolic and consequent physiological alterations (Fig. 8). Disappearance of microvilli associated with the CPV-infected midgut could contribute to altered nutrient absorption by larvae. The most frequent physiological perturbations noted in the infected insects are a progressive cessation of larval feeding, extension of the larval stage duration, smaller and lighter larvae and pupae, adult and pupal malformations and lower viability, decrease in adult fecundity, as well as transmission of the virus to the offspring and reduction of the level of progeny insect populations.

CPV-infected insects are more sensitive to detrimental environmental stresses. High temperature has an inhibitory effect on virus replication but low temperature treatment activates endogenous CPV replication and has detrimental effects on the survival of an insect population infected by CPV. In pilot studies, for example, we observed a higher mortality of *E. scandens* larvae treated with *Es* CPV following a rapid and exceptional decrease in temperature during one summer (unpublished results). In other studies, Katagiri [16] proved that CPV-infected *Dendrolimus spectabilis* larvae, compared to healthy populations, were less resistant in the forest to hibernation which resulted in a high mortality rate of CPV treated insects in Japan. Moreover, an analysis of results obtained on differential survival rates following hibernation in different climatic conditions in China of CPV-infected *Dendrolimus*, could indicate a lower survival of infected insects in a colder environment [17].

The reasons for this decrease of resistance are unknown. One of the physiological imbalances due to CPV infection is an extension of larval stage duration which therefore results in hibernation of larvae at non favorable stages. It can not be excluded also that physiological and metabolic alterations in larvae due to CPV infection will affect the synthesis of a cryoprotectant which will explain the high mortality rates in over wintering CPV-infected insects.

Table1. Effect of CPV application (10^4) polyhedra/larva (laboratory studies)

Neonate larvae

	Infection (%)	Mortality (%)	Food consumption (mg)	lenght (mm)
Treated	76.8	52.48	2.32+/-1.68b	9.62+/-1.38b
Control	0	10.17	39.92+/-14.37a	23.54+/-2.72a

2ND Instar

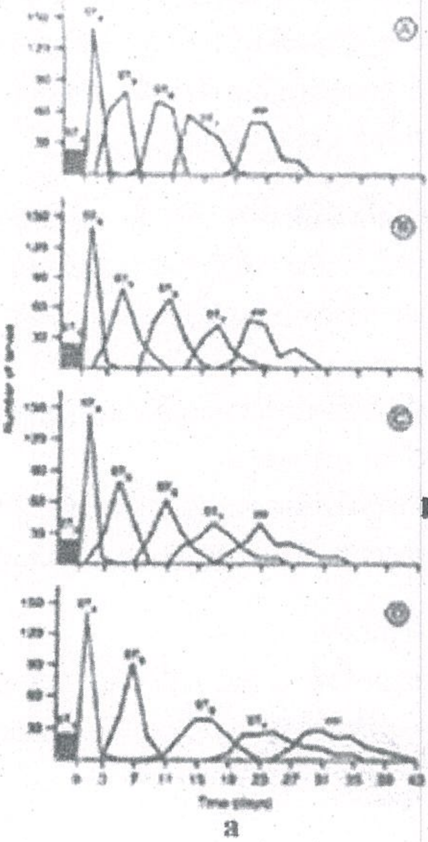
	Infection (%)	Mortality (%)	Food consumption (mg)	lenght (mm)
Treated	100	56.63	1.25+/-2.41b	7.92+/-1.38b
Control	0	6.30	8.11+/- 4.25a	10.42+/-1.48a

Table 2. Effect of CPV application on larvae - 2nd instar- (Field studies)

Doses	Infection (%)	Mortality (%)
CONTROL	0 a	52.70 a
$5x10^1$	26.8 b	53.55 a
$5x10^4$	25.8 b	74.70 b
$5x10^6$	96.3 c	94.10 c
$5x10^7$	100.0 c	95.00 c

Persistence of the infection

CPV's are important pathogens found frequently in laboratory-reared insects [18, 19, 20]. Their infection can lead to loss of insect productivity and the decline of an entire insect colony over several generations. The mechanisms by which CPV infections persist in an insect colony are partially known. But horizontal transmission of the virus is quite well documented. Feces contaminated with viral polyhedra are the most important instrument maintaining the virus infection in an insect population. Meconium of insects are CPV-infected [21, 22, 23] and therefore the eggs are easily surface-contaminated with viral polyhedra. As mentioned earlier, the transmission of viral particles from infected cells to newly regenerated differentiated midgut cells is another known mechanism for viral persistence. However, numerous experiments and



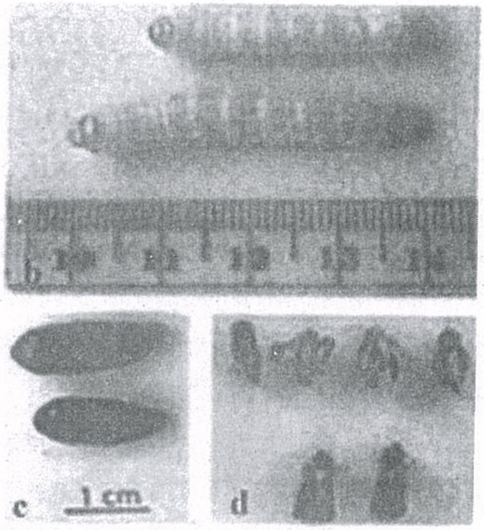


Figure 8 Some physiolociacl perurbations caused by CPV infection
a: Delays in development of *E.scandens* larvae infected with CPV at third instar
A: control, B,C and D : 1000 , 100000 and 5x1000000 polyhedra/larvaerespectively . ST: larval stage (3,4,5,6,7). ST: PP prepupa.
b, c d: Effects on larval and pupal lenght, malformation of infected adults.

observations suggest that the viral infection could also be transmitted by a trans-ovum mechanism and be latent in the insect (Fig. 9) Many endogenous and exogenous factors were demonstrated to derepress CPV infection in an insect colony. It is not known in which state or cells the virus persists. The detection of the CPV genome and transcripts will be very helpful for elucidation of this phenomenon and for studying the outbreak of CPV in insect populations.

The use also of insect cell systems permitted the demonstration that the persistence of viral infection *in vitro* is possible. Depending on the CPV strain, 15 to more than 100 passages of CPV-infected H. armigera cells were possible during which polyhedrin was continuously (Fig. 10).

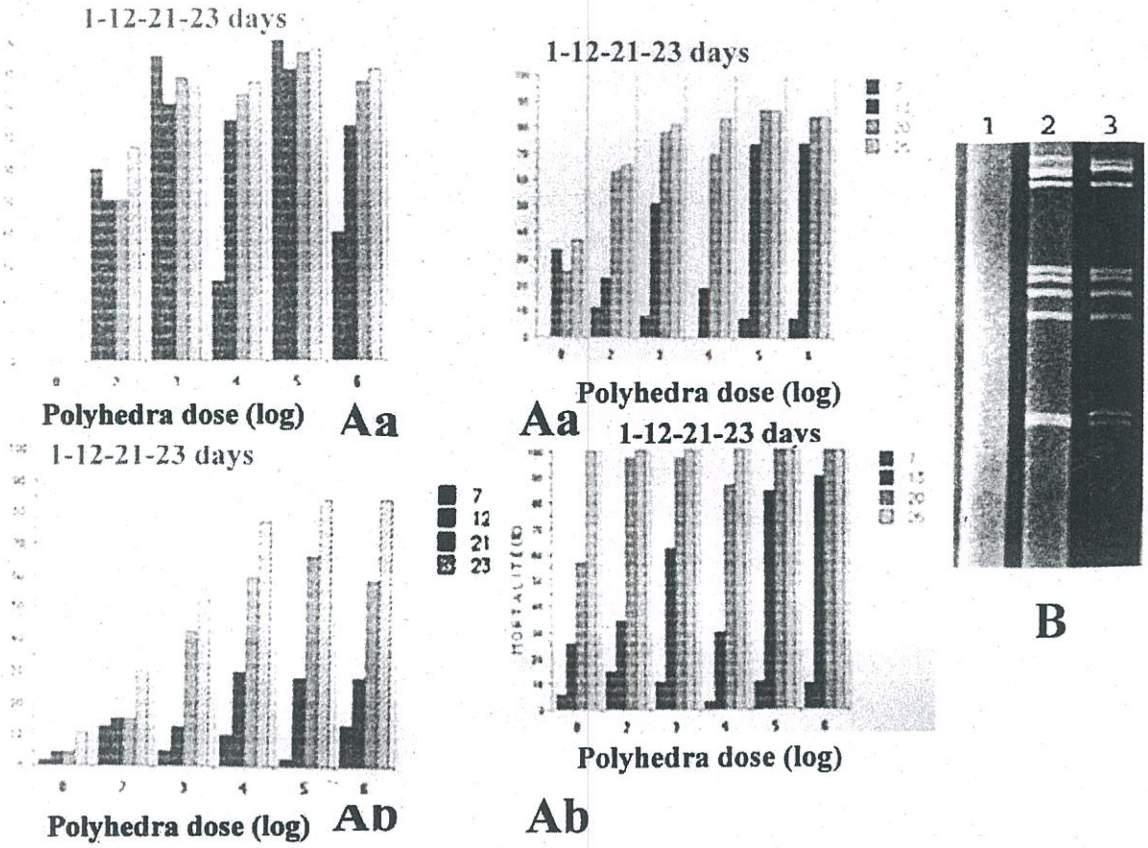


Figure 9 Latency of CPV ; Efficacy of CPV treatments on the spruce budworm colony chronically infected with CPV.

A: Infection (a) and mortality (b)

B: Derepression of the spruce budworm CPV in a colony.

1. Control.
2. Injection of sterile distilled water into larvae and analysis of the larvae after one week. Detection of dsRNA of the spruce budworm CPV.
3. dsRNA of the spruce budworm CPV .

Synergetic interactions with other insect viruses, microbial toxins and chemicals, increase susceptibility of CPV-infected insects to pathogens

Synergistic interactions between CPV and permethrin, a current chemical insecticide used in pest control have been demonstrated in the laboratory [24] An higher mortality rate was obtained in CPV-infected insect populations treated with permethrin and less chemical was needed to achieve the same mortality level, if the insect was viral infected (Fig. 11) [24]. This synergistic interaction was demonstrated also when larvae were NPV-infected. It will be of interest to determine if CPV infection of larvae predisposes insects to pesticide susceptibility and enhances the effects of other detrimental environmental conditions by means of metabolic and physiological alterations associated with CPV infection. The association of CPV with microsporidia, which has similar physiological effects on insects is not well documented. To our knowledge, the only observation of such a dual infection was made when *Es* CPV was first isolated [8] along with a microsporidia. Despite the fact that larvae reared in the laboratory were heavily CPV-infected, the development of microsporidia in the same CPV-infected cell was noted without, however, an increase in larval mortality rate.

The physiological alterations and mortalities noted when insects are infected by CPV are enhanced significantly when bacteria such as *P. maltophilia, B. subtilis, E. coli, S. epidermidis* are present as contaminant [25]. We have also demonstrated in our laboratory that CPV-infected *C. fumiferana* larvae were more susceptible to bacterial contamination and that synergistic interactions in relation with insect mortality were obtained between CPV and *B. thuringiensis* infections (Table 3). It is also suggested that endogenous bacteria develop better at the low pH of the midgut, produced as a result of CPV infection and cause together with CPV infection a larger detrimental effect on larvae [26]. In the same way an increased mortality in *Dendrolimus* treated in the forest with a mixture of CPV and *B. thuringiensis* compared to a single pathogen application was demonstrated [16,17]. Some types and strains of CPVs are lethal but a cytoplasmic polyhedrosis is mainly chronic, persistant and slow in evolution. In contrast, NPV is more commonly lethal. A combination of the two pathogens will have therefore some interesting practical applications in both short and long term biological control of insects. Different observations in experimental fields demonstrated a contamination of NPV with CPV together with a cohabitation of the two viruses in natural *P. unipunctata* populations over many years with

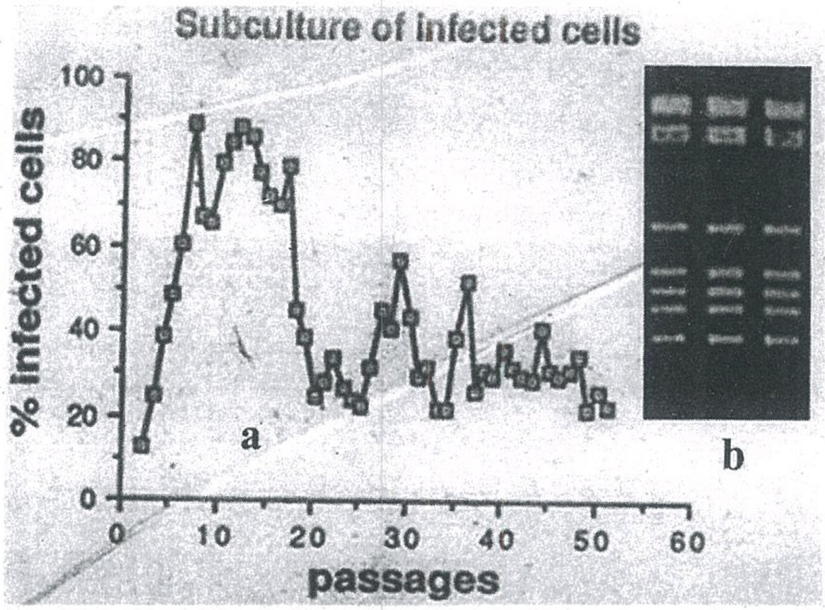


Figure 10 Persistence of CPV (Ha CPV type 14, chinese strain) in Ha cell line. Detection of polyhedra and viral ds RNA in the infected cells through the passages. a: polyhedra b: dsRNA

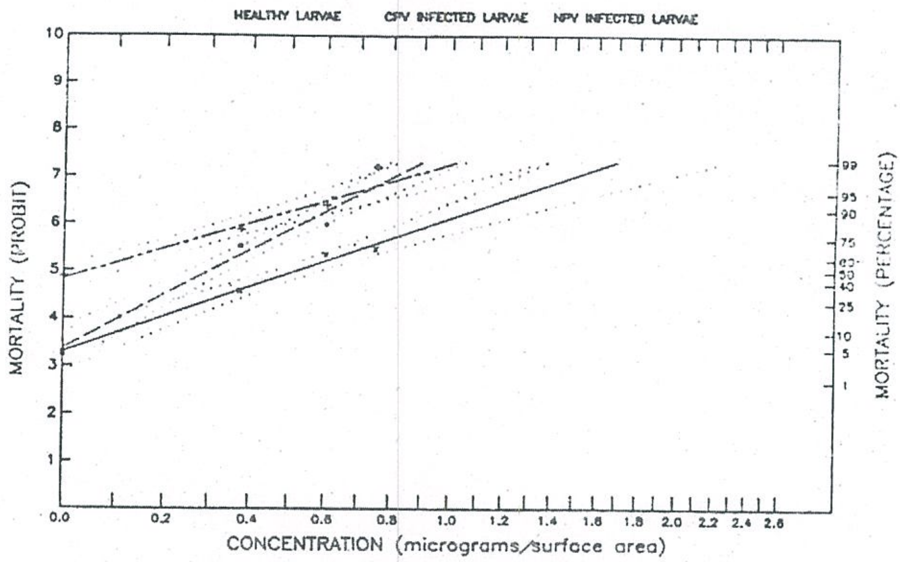


Fig 11 Effects CPV and NPV infection on permethrin-treated larvae.

however predominance of CPV. Other epidemiological studies demonstrated on several years the occurrence of the two viruses in *Lymantria fumida*. Some laboratory studies have been conducted on the relations between CPV and NPV in different insects such as *Pyrausta cardui*, *Choristoneura fumiferana* and *Malacosoma disstria* [27]. Several conclusions have been drawn on the effects of CPV contaminating a NPV inoculum, but no clear feature of the interactions has been presented. Interference as well as synergetic interactions have been demonstrated. It is important to point out that several parameters as the sequence of applications, the ratio of the two viruses and larval development, influence the nature of the interactions. Several observations [28, Cunningham, personal communication] indicate that contamination of NPV with CPV increases the efficacy and lethality of NPV. Moreover, studies conducted in our laboratory demonstrated that lethality and rapidity of action of *Agrotis segetum* NPV were increased significantly if *E. scandens* larvae were previously infected with CPV (Table 4) Lethality half-,times as well as mortality rates, of larvae were significantly improved following these dual treatments [29].

A simultaneous infection of *B. mori* cell lines with *Es.* CPV and *Bm* NPV has been achieved but CPV and NPV were not observed in the same cell (unpublished observations). However, a dual infection of *Es* CPV and *Galleria mellonella* NPV and the replication of the two viruses in a single cell were demonstrated [30]. However, Interference between the two viruses was noted during the process of baculovirion inclusion in polyhedra and the synthesis of the NPV particle membrane. Hypersynthesis and crystallization of polyhedrin, which characterizes the *Es* CPV strain, was suspected as cause of this interference. However when the *Es* CPV polyhedrin was expressed using a baculovirus expression system, no negative effect of the replication of the baculovirus or the expression of CPV polyhedrin and the maturation of polyhedra was observed (Fig. 7). It was also suggested that an interferon like substance may be produced by CPV which interferes with Baculoviral replication [30]. If such phenomenom occurs it will affect NPV replication *in vivo*. We noted however synergistic interaction in [29].

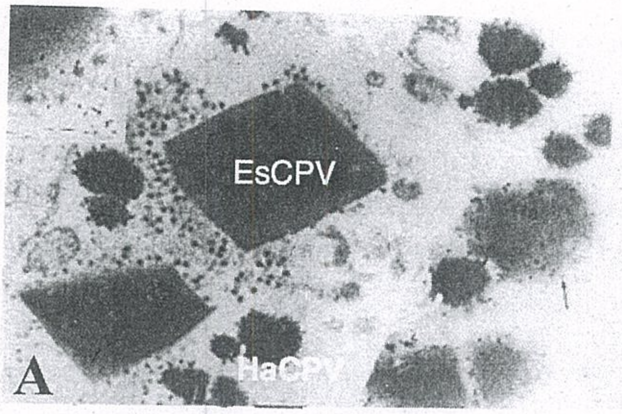
The effect on CPV replication of another DNA virus has also been studied. No synergism or interference was noted when larvae of *E. scandens* were infected with *Chilo* iridescent virus (CIV) and *Es* CPV (unpublished results). However, interestingly, significant enhancement of the infection rate and the number of polyhedra of *Es* CPV, were obtained when these two viruses replicated together in the cytoplasm of *C. fumiferana* 124 cell line [31] (Fig. 12).

Table 3. Mortality of larvae treated with NPV plus CPV

NPV	**CPV**	**Mortality**
0	0	0%
10^1	0	0
10^2	0	2.1
10^3	0	6.5
10^4	0	49.1
0	10^5	40.4
10^1	10^5	27.3
10^2	10^5	61.7
10^3	10^5	88.3
10^4	10^5	93.2

Table 4. Effects of combinations of Bt and CPV on the spruce budworm *Choristoneura fumiferana*.

TREATMENT	**MORTALITY 7 DAYS**	**MORTALITY 14 DAYS**	**ADULT EMERGENCE**
Control	0.00	5.88	10
	9.90	21.20	15
	0.00 *	5.88 *	10*
Bt 0.42 UI/L	8.82	26.50	7
	11.10	25.00	16
	8.22 *	26.50 *	7*
Bt 0.42 UI/L +CPV30 P/L	15.20	42.40	4
	17.10	40.00	8
Bt 0.42 UI/L +CPV 3P/L	14.30	57.10	5
CPV 30P/L	9.03	36.40	8
	11.10	40.00	8
CPV 3P/L	0.00 *	34.10 *	11*



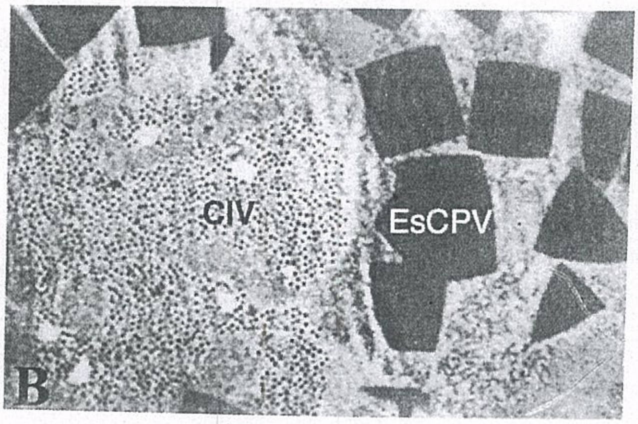


Figure 12 Double *in vitro* infectio by two cytoplasmic viruses.
A: Ha CPV and EsCPV
B: Es CPV and CIV

The enhancement was induced by the replication and not by the components of the cytoplasmic DNA virus.

Use of cytoplasmic polyhedrosis viruses in biological control of insects

Cytoplasmic polyhedrosis viruses have received, in the past, less interest than baculoviruses as biological control agents. The main reasons are their lower lethality to larvae and the slower action of CPV's compared to nuclear polyhedrosis viruses. Another reason, for safety consideration, is their taxonomic relatedness to other vertebrate Reoviridae. However, as has been shown in this paper, CPVs are very infectious, persistent and cause high larval mortality rates in synergy with various exogenous natural factors such as bacterial contaminants and pathogens, climatic conditions, chemical, etc. Metabolic and physiological alterations in surviving insects induced by CPV infection cause also a decrease of larval consumption (Tables 1and 2) combined with an overall high mortalities over an entire generation of insects CPV's will be more useful for plant protection in stable ecosystems such as forests and when a certain level of damage can be tolerated [14]. Examples of good control of insects with CPV, cited in the review of Granados [32] include those of *Lymantria dispar* and *fumida* in Japan, *Thaumetopoea pityocampa* in France and *Trichoplusia ni* in USA with their respective CPV. In addition to this list, CPV's of *Dendrolimus pini* against *D. pini* and *Malacosoma neustria* showed promising control actions. Up to now only the CPV of *Dendrolimus spectabilis* has been commercialized in Japan as Matsukemin product [33,16]. Efficacy of this virus alone, or synergistically with *Bt.*, has been demonstrated in Japanese forests [34,35]. Moreover, in China, CPV together with *Bt* have been used from more than 20 years on a large scale against *Dendrolimus* with satisfactory results [36,37, 17]. In Canada, despite the efficacy of spruce budworm and forest tent caterpillar CPV's suggesting their possible use in biological control, no field utilization of these viruses have been made. However, work on *C. fumiferana* CPV has demonstrated the efficacy of this CPV [5] alone or in synergy with *Bt.* [20]. Therefore, the CPV of spruce budworm could in future be another example of the importance of CPV as a viral insecticide in forests.

2. CONCLUSION

Despite the fact that CPV's received less attention than the baculoviruses and that a relatively limited number of laboratories around the world have been involved in research projects on these viruses, important fundamental data on their replication *in vivo* and in insect cells cultivated *in vitro* as well on the structure of the virus particles and their molecular biology have been obtained. Moreover; as summarized in Fig.13, the cytoplasmic polyhedrosis viruses represent interesting and peculiar models of interaction with the hosts and natural environmental conditions

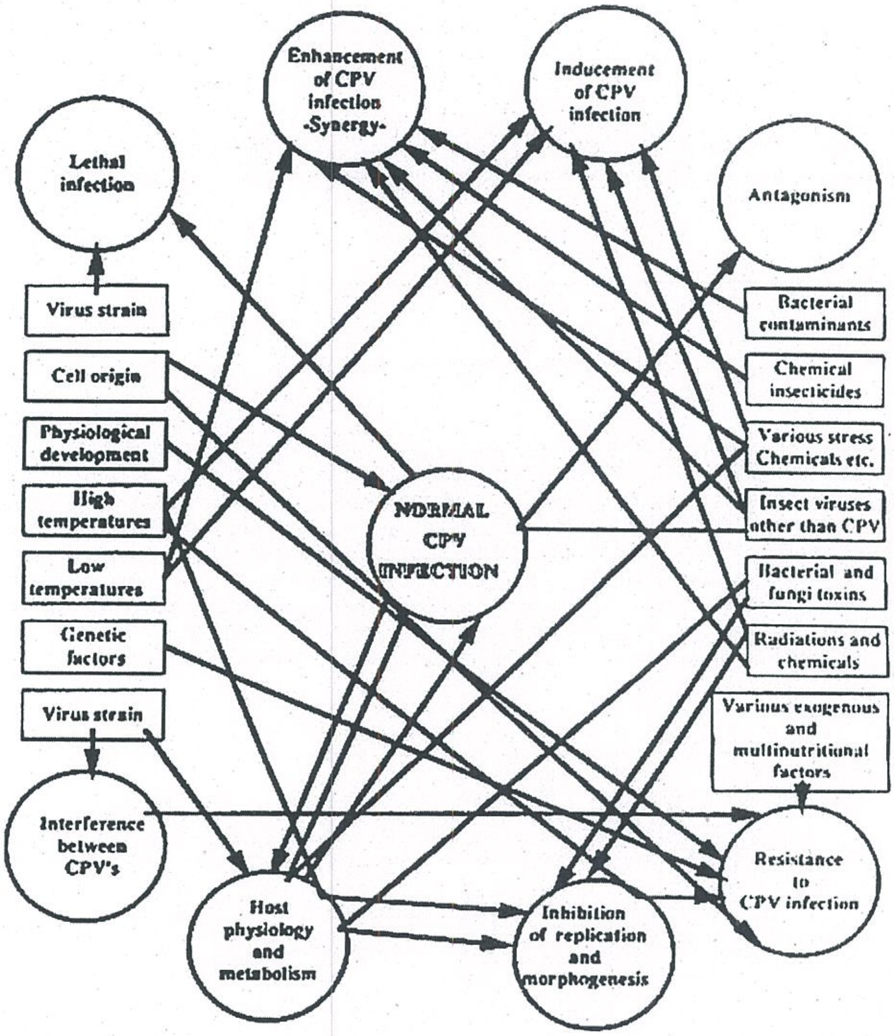


Figure 13 Complexity of interactions between endogenous and exogenous factor effects on the evolution of CPV infection and pathogenesis. From S. Belloncik, 1996

which can be exploited in biological control of insects programs as well as in animal biology and physiology researches.

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