Vertical Distribution of Nuclear Polyhedrosis Virus-Infected Gypsy Moth (Lepidoptera: Lymantriidae) Larvae and Effects on Sampling for Estimation of Disease Prevalence

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ABSTRACT
The vertical distribution of nuclear polyhedrosis virus (NPV)-infected gypsy moth, Lymantria dispar (L.), larvae in natural and artificial infestations was examined; two methods that use burlap bands to estimate the incidence of NPV in gypsy moth populations were evaluated. We found that infected larvae tend to aggregate in the upper portions of trees before death. Because of this infection-induced behavioral effect, burlap band–based methods may tend to underestimate the incidence of NPV in gypsy moth populations.

KEY WORDS
Lymantria dispar, nuclear polyhedrosis virus, sampling

DISEASE-RELATED BEHAVIORAL abnormalities have been described for a number of insect/pathogen associations. For instance, sawflies that normally feed gregariously may switch to solitary feeding habits or may wander away from a food source when infected with some viruses (Smirnoff 1965). Many insects infected with Entomophthorales fungi characteristically climb to the top of the host plant (Carruthers & Soper 1987, Entwistle & Evans 1987). A similar behavioral effect termed “Wipfelkrankheit” or “tree-top disease” has also been described for a gypsy moth [Lymantria dispar (L.)] conspecific, the nun moth, L. monacha (L.), when infected with nuclear polyhedrosis virus (NPV) (Komarek & Breindl 1924).

Such behavioral effects could alter the distribution of infected individuals in a population. To estimate the prevalence of disease in a population as an aid in pest suppression and pest management decision making or to monitor and evaluate the efficacy of microbial insecticides, a sampling scheme based on the distribution of infected and healthy individuals in a population must be developed. However, the effects of NPV infection on behavior and distribution of gypsy moth larvae have not been examined previously. In addition, sampling methods for estimating disease prevalence in gypsy moth populations have not been standardized.

Collection and rearing of insects with subsequent monitoring for causes and rates of mortality is a method that has been used to estimate pathogen levels in a population (Woods & Elkinton 1987, Woods et al. 1991). Unfortunately, locating sufficient numbers of gypsy moth larvae in a forest in low and even moderate-density populations is difficult, particularly during late larval stages when larvae spend daylight hours resting in cryptic places (Forbush & Fernald 1896). Burlap bands wrapped around tree boles provide artificial resting sites highly favored by late instars and have been used to facilitate collection of larvae for pest control (Forbush & Fernald 1996) and to detect and monitor gypsy moth populations (McManus et al. 1980, McManus & Smith 1984).

Burlap bands have also been used to estimate larval mortality rates due to various causes, including NPV (Webb et al. 1989, 1990). Although use of burlap bands in this manner greatly simplifies sampling, whether sampling from burlap bands alone provides a reliable or accurate estimate of the effect of NPV on a population is not known. To develop a sampling protocol that provides an unbiased estimate of disease prevalence, the distribution of infected individuals within a population must be known.

Our study was done to determine the vertical distribution of NPV-infected gypsy moth larvae in forested sites and to compare the use of burlap bands with a more extensive collection method used to estimate the incidence of NPV in gypsy moth populations.
Materials and Methods

We observed the movement and vertical distribution of gypsy moth larvae to determine whether distribution of larvae is altered by NPV infection and whether NPV-infected and healthy larvae use burlap bands similarly as daytime resting sites. In addition, we examined two methods for use of burlap bands to estimate disease prevalence: (1) counting the number of live and dead larvae found under burlap bands and (2) rearing larvae collected from under burlap bands. Estimates obtained by these two methods were compared with each other and with the prevalence estimated by rearing larvae extensively collected from the same sites.

Behavioral Observations of Infected and Uninfected Larvae. Behavioral observations of infected and uninfected larvae were done to determine experimentally if NPV infection induces changes in larval movements that could result in altered vertical distribution of infected larvae.

At a site located on Cape Cod, MA, which harbored a very low resident gypsy moth population, scaffolding was erected around a black oak tree (Quercus velutina Lam.), 6 m tall, 12.5 cm diameter at breast height (DBH). This site was chosen because resident gypsy moth populations had been low for a number of years; therefore, the levels of NPV inoculum persisting in the area were also expected to be quite low. Adjacent trees were trimmed so that their branches did not contact the observation tree; some of the understory vegetation was removed to facilitate human movement around the base of the tree. Two burlap bands were wrapped at 1 and 1.3 m height around the bole of the observation tree. In addition, burlap bands were also wrapped around adjacent trees to aid in the collection of wild larvae and detection of movement of released larvae between trees.

New Jersey Strain (Bell et al. 1981) larvae were reared in cups of artificial diet that were held in an outdoor insectary. Within 24 h of molting to the third or fourth stadium, larvae were infected with NPV (Gypchek) by feeding each larva a cube of artificial diet (≈27 mm³) contaminated with a 10-µl droplet containing ≈10⁶ polyhedral inclusion bodies (PIB) in sterile distilled water. Control larvae were fed a cube treated with 10 µl of sterile distilled water. Larvae that failed to consume the entire cube were excluded from the experiment. Larvae fed virus and controls were transferred to separate outdoor cages containing bouquets of black oak foliage held in jars of water. The foliage in these cages was first disinfected by soaking for 30 min in 0.05% sodium hypochlorite, followed by a 30-min soak in water. Foliage was removed and replaced with fresh foliage every 3–4 d.

Within 48 h after they molted to the next stadium (fourth or fifth), larvae were collected from the cages and were marked on the dorsum with a spot of fluorescent powder to distinguish the treated from the control larvae. Larvae from both treatments were released between 0800 and 1600 h onto the observation tree by placing them in a paper cup that was attached to the bole of the observation tree underneath a burlap band. Infected larvae died 5–10 d after release. As an additional control, some of the marked larvae from each treatment were not released but were transferred to 30-ml cups containing artificial diet. They were kept in an outdoor insectary where their time and cause of death were monitored twice weekly.

Wild larvae that were collected from the area near the observation tree were also marked uniquely with fluorescent powder and were released along with the infected and uninfected laboratory larvae, to serve as a control for normal movement and activity. Infected, control, and wild larvae were released in groups of 40 to 80 on each of five different occasions. In the first release, larvae were infected 6–7 d before release, whereas all subsequent releases were done at 10–11 d after infection. A total of 121 treated, 125 control, and 44 wild larvae was released.

We observed the released larvae at periodic intervals after release and at several different times of the day and night. At each observation, the observers systematically searched the entire tree by climbing on the scaffolding that surrounded the tree on all sides. The location of each larva in the tree, its color marking, and its activity (feeding, resting, walking, or dead) were recorded. Each dead larva was collected into an empty plastic container that was marked with the date and the location on the tree where it was found. The cadaver was taken to the laboratory where it was examined microscopically for the presence of PIBs to determine if death was due to virus infection.

Cadaver Collections. Because infected laboratory larvae died at the tree top, a wild population was sampled to determine if naturally occurring NPV-killed gypsy moth larvae also aggregate at the tops of trees. Several white oak (Q. alba L.) and black oak trees located in site DPW were climbed and searched for larval cadavers. Trees were divided into four height classes (0–1.7 m, 1.8–3.3 m, 3.4–5.0 m, and 5.1–6.3 m). Each cadaver found on these trees was collected into a 30-ml container labelled with the height class and location in the tree where it was found; it was taken to the laboratory for microscopic examination for the presence of PIBs. Each cadaver was scored as either virus-killed, if a large number of PIBs were found (average of ≈10 PIBs per microscopic field in three fields), or not virus-killed, if few or no PIBs were found.

Evaluation of Burlap Bands for Estimation of Disease Prevalence. Study Sites. The study was done at three forested sites on Cape Cod, over
two consecutive seasons. In 1986, two 9-ha sites were established. Gypsy moth population density was estimated by counting all egg masses within each of 169 5-m fixed-radius plots (Kolodny-Hirsch 1986) that were arranged in a 13 by 13 grid throughout each site. One site (Otis 1) harbored a high-density gypsy moth population (mean ± SEM = 2,994 ± 26 egg masses per ha); the second site (Otis 5) supported a moderately low-density population (311 ± 10 egg masses per ha). Each site was divided into a three-by-three grid of nine 1-ha plots. Each plot was further divided into a four-by-four grid of 16 625-m² subplots. At each of three randomly selected subplots within each of the nine plots at both sites, burlap bands were stapled around each of 11 trees closest to the subplot center. Selection of trees was based only on location at the center of the subplot (trees measuring smaller than 7 cm DBH were excluded).

In 1987, the study was conducted at a third site (DFW) 1.5 ha in area. Egg masses counted within 15 5-m-radius plots indicated that this site supported a very high-density gypsy moth population (3,574 ± 265 egg masses per ha). Ten burlap subplots and 15 subplots without burlap, measuring 625 m² each and arranged in an alternating checkerboard pattern, were established within this site. At the center of each of the subplots chosen for burlap sampling, burlap bands were stapled around the bole of each of 10 trees. The 10 centernmost trees were banded with burlap regardless of species, and trees smaller than 7 cm DBH were excluded.

**Larval Collections Under Burlap Bands.** To determine the vertical distribution of infected larvae and to compare NPV prevalence among larvae found under burlap bands with that among larvae sampled more extensively, larvae were collected three times at weekly (Otis 5) or biweekly (Otis 1) intervals in 1986, beginning at the peak of the fourth stadium. Each time, larvae were collected from one burlap subplot and an adjacent subplot without burlap within each plot at each site. To avoid the possibility that repeated sampling of the same burlap bands might affect estimates, only one burlap subplot within each plot at each site was sampled each time. In this manner, each burlap band and each subplot were sampled only once. Eight randomly selected larvae (four larvae from each of both sides of the tree) were collected from under each burlap band. At the center of each adjacent subplot without burlap, the understory vegetation, litter, tree trunks, and crowns were searched. Pole-pruners and ladders were used to search the crowns and upper tree trunks until approximately four larvae were found and collected from each subplot. The approximate height from which each larva was collected was recorded.

After collection, each larva was placed into a 30-ml cup containing 10 ml of artificial diet (Bell et al. 1981), and the lid was marked to identify the location and date of collection. Larvae were held in an outdoor insectary. All larvae collected from under burlap bands and other strata were observed weekly, and the apparent cause and date of mortality were recorded for each larva. Cause of death was attributed to NPV if a large number of PIBs were observed by microscopic examination of cadaver tissues. We used the method of Woods & Elkinton (1987) to estimate weekly NPV-caused mortality within each population from the percentage of mortality among collected larvae. The proportion of larvae collected from under burlap bands that subsequently died within 8 d because of NPV infection was compared with percentage of larvae that died within 8 d because of NPV infection among those collected from each stratum and from all other strata combined. Cumulative mortality was calculated as 1 minus the product of the weekly probabilities of survival (Woods et al. 1991).

**Comparison of Single Versus Repeated Sampling of Burlap Bands.** To compare estimates obtained by repeatedly sampling burlap bands with those from sampling each band only once, we resampled one burlap band in each subplot each week. Each time, eight larvae were collected from each band and reared as described above. Larvae remaining under each band were destructively removed from the tree with a wire brush. NPV-caused mortality among larvae collected from these repeatedly sampled bands was compared with that among larvae collected at the same time from under burlap bands previously sampled in the same subplots.

**Larval Counts Under Burlap Bands.** In 1986, 12 additional points were established within each site and the number of live and NPV-killed larvae found under burlap bands were counted. These points were uniformly distributed throughout each site in a three-by-four grid. At each point, burlap bands were stapled around each of 25–35 trees. Counts were done three times in the Otis 5 site, at approximately the peak of the fourth, early fifth, and peak of the fifth larval stadium. The Otis 1 site was counted once (at the peak of the fourth stadium). The counts were done by a previously developed sampling protocol (C. Jones, Carey Arboretum, New York, personal communication). On each occasion, each burlap band was lifted and the number and stadium of all live gypsy moth larvae found under-
neath each burlap band were recorded. In addition, the number, stadia, and probable cause of mortality of all dead larvae were recorded. Cadavers killed by NPV were identified on the basis of gross morphological appearance (i.e., flaccid, inverted or inverted-V posture, milky brown liquid oozing from the cadaver, or a combination of these characteristics). The proportion of larvae killed by NPV was calculated as the number of NPV-killed larvae divided by the total number of larvae (dead plus live) counted under each burlap band.

Data Analyses. The patterns of virus-caused mortality observed among larvae collected from burlap bands and from all other forest strata (litter, understory, trunk, or crown) over the 3–5-wk periods were compared with the expected mortalities by \( \chi^2 \) tests (Steel & Torrie 1980). The effect of repeated burlap sampling was examined by analysis of variance. Arcsine, square root transformed proportions of NPV-killed larvae collected from repeatedly and singly sampled burlap bands were analyzed (PROC GLM; SAS Institute 1987). Logit regression (SAS PROC CATMOD; SAS Institute 1987) was done to determine if height class was a significant factor affecting the incidence of NPV among collected cadavers. The effects of height, date, and stratum of collected larvae on their subsequent mortality rates were also examined (ANCOVA; SAS PROC GLM; SAS Institute 1987) with height as a covariate. Variation in activity and height class of released virus-infected and uninfected larvae was analyzed by ANOVA (SAS PROC GLM). Mean separations were determined by Sidak’s inequality test (SAS Institute 1987).

Results

Behavioral Observations. Observations of infected laboratory larvae released onto a tree banded with burlap indicated that, before death, infected larvae were found significantly less frequently under burlap bands than were healthy larvae (\( \chi^2 = 49.3; \) df = 1; \( P < 0.0001 \)). The effect of disease condition (infected versus healthy) was a significant factor affecting height at which larvae were found. Infected individuals occurred significantly higher (\( P = 0.0001 \)) in the tree than healthy larvae (Fig. 1). However, at night, both infected and healthy insects were found primarily in the tree crown. Both treated and control laboratory larvae occurred significantly higher (\( P < 0.05 \)) in the tree than did the wild larvae released at the same time.

Infected larvae tended to die at tree top. Most infected larvae died in the top quarter of the tree, and almost half died in the top 30 cm of the crown (Fig. 2). Mortality among the control
Disease Prevalence. Larval Collections Under Burlap Bands. Disease prevalence was significantly greater in the upper tree canopy ($\chi^2 = 7.35; df = 1; P = 0.0067$) (Fig. 3). Postmortem dissection of 180 naturally occurring wild cadavers collected from trees showed that 39 larvae (21.7%) died because of NPV infection.

Evaluation of Burlap Bands for Estimation of Disease Prevalence. Larval Collections Under Burlap Bands. Burlap band sampling frequency (repeated versus single sampling) was not a significant factor affecting the proportion of larvae dying of NPV infection ($F = 0.04; df = 1; P = 0.83$) (Table 1). Because this observation was consistent at both sites, repeatedly and singly sampled bands were pooled for all further data analyses, and the experimental protocol was altered the following year so that all bands were sampled repeatedly.

The strata from which larvae were collected had a significant effect on the levels of larval NPV-caused mortality in all of the plots (Otis 1: $F = 3.93; df = 4; P = 0.005$. Otis 5: $F = 5.64; df = 4; P = 0.0001$. DPW: $F = 2.79; df = 4; P = 0.03$) (Table 2). Only one stratum (bole) had significantly higher mortality than other strata, and it was not significantly different from NPV mortality among larvae collected from under burlap bands in any of the plots ($P > 0.05$). However, when cumulative mortality among larvae collected from all other strata within each site was pooled, the pooled cumulative mortality was significantly higher ($P < 0.05$) than that among larvae collected from under burlap bands alone in two of the three plots (Otis 5 and DPW) (Fig. 4).

When NPV-caused mortality among larvae was stratified by the height from which larvae were collected, height was not a significant factor ($F = 0.11; df = 1; P = 0.74$). Although the highest mortality occurred among larvae collected from the highest stratum (>6 m), this value was not significantly higher than that of any other stratum ($P > 0.05$), nor was it higher than those of the other strata combined ($F = 2.05; df = 1; P = 0.15$).

Larval Counts Under Burlap Bands. The results of mortality estimates based on visual counts of the number of live and dead larvae found under burlap bands in Otis 1 and Otis 5 are also shown in Fig. 4. Virus mortality, as determined by this method, was significantly lower in both plots compared with estimates obtained by rearing larvae collected from all strata (Otis 1: $\chi^2 = 68.45; df = 1; P \leq 0.05$. Otis 5: $\chi^2 = 78.67; df = 1; P \leq 0.05$).

Discussion

Previous studies indicate some disagreement concerning behavioral effects associated with NPV infection in the gypsy moth. Doane (1967) reported that partial paralysis, reflected in reduced control of proleg crochet movement, occurred in infected larvae a few days before death, and that feeding ceased before other external symptoms of infection were expressed. Although

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Table 1. Mean percentage of NPV-induced mortality among larvae reared subsequent to their collection from burlap bands that had been previously sampled (repeat) or were sampled for the first time

<table>
<thead>
<tr>
<th>Sampling frequency</th>
<th>% Mortality (SE)</th>
<th>No. bands sampled</th>
<th>No. larvae collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plot 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeat</td>
<td>11.5* (1.8)</td>
<td>27</td>
<td>401</td>
</tr>
<tr>
<td>First time</td>
<td>9.7 (1.2)</td>
<td>279</td>
<td>1,065</td>
</tr>
<tr>
<td>Plot 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeat</td>
<td>8.8 (2.1)</td>
<td>27</td>
<td>269</td>
</tr>
<tr>
<td>First time</td>
<td>4.6 (0.5)</td>
<td>279</td>
<td>984</td>
</tr>
</tbody>
</table>

* Percentage of NPV-induced mortality was not significantly affected by sampling frequency ($F = 0.04; df = 1; P = 0.83$).

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Table 2. Cumulative proportion of larvae dying of NPV subsequent to their collection from different forest strata and from burlap bands at three sites

<table>
<thead>
<tr>
<th>Stratum</th>
<th>DPW Cumulative proportion NPV-killed (n)*</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cumulative proportion NPV-killed (n)</td>
<td>Otis 1</td>
</tr>
<tr>
<td>Litter and understory</td>
<td>0.55a&lt;sup&gt;b&lt;/sup&gt; (202)</td>
<td>0.21a (498)</td>
</tr>
<tr>
<td>Tree bole (except under burlap)</td>
<td>0.49a (363)</td>
<td>0.37b (564)</td>
</tr>
<tr>
<td>Crown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>0.56a (215)</td>
<td>0.13a (132)</td>
</tr>
<tr>
<td>Branches</td>
<td>0.56a (101)</td>
<td>0.19a (257)</td>
</tr>
<tr>
<td>Burlap</td>
<td>0.52a (1,322)</td>
<td>0.21ab (1,451)</td>
</tr>
</tbody>
</table>

* Total number of larvae reared.
<sup>b</sup> Proportions within the same column followed by the same letter are not significantly different ($P > 0.05$; Sidak’s inequality test [SAS Institute 1987]).
effects on locomotory capabilities were not reported, movement might not be seriously affected by such partial paralysis. Lance et al. (1986) found no differences in feeding rhythms of infected and healthy larvae, although they did not indicate how advanced infection was at the time of observation. Liebhold et al. (1986) found no difference in the tendency of larvae that subsequently died from NPV infection to move between trees compared with larvae that did not die from NPV.

Although we did not find rates of virus infection to be higher in natural populations among live larvae collected from the upper tree crowns compared with larvae collected from lower strata, evidence from other aspects of our study suggest that infected larvae tend to climb to the tree top before death. This behavioral shift was evident among released infected larvae that stayed higher in the canopy while still alive compared with uninfected larvae (Fig. 1) and infected larvae that occurred at tree top after death (Fig. 2). The same shift in behavior was observed among virus-killed cadavers from the natural population that occurred higher than larvae dying of other causes (Fig. 3). In high-density populations, some of the larvae remain in the canopy of trees and do not seek daytime resting locations on the lower bole or in the litter (Lance et al. 1987). It is possible that these larvae are also more susceptible to infection or more likely to encounter NPV than larvae that leave the canopy during the day. However, such a phenomenon would not explain why larvae that were exposed to NPV in the laboratory climbed to the tree top but uninfected larvae did not. Our results indicate that infection results in a behavioral shift that causes larvae to seek elevated locations before death.

Evans & Entwistle (1987) suggested that infection of nerve ganglia and hemocytes starvation, causing insects to search continually for food. This explanation is consistent with our finding that a greater proportion of infected larvae were found in the crown of the tree during daylight hours, compared with uninfected larvae. Although we did not find that actual feeding activity was altered in infected individuals, NPV could cause larvae to search for food during the day without stimulating feeding activity. The specific mechanisms causing infected larvae to seek elevated sites are yet to be elucidated.

Both the infected and uninfected laboratory larvae released for observation were found higher in the tree compared with wild larvae released at the same time. To minimize any behavioral effects that might have arisen because of handling, these wild larvae were collected from burlap bands on adjacent trees just before release on the observation tree. Therefore, uncontrollable differences such as in phenological development, genetics, or levels of parasitism or disease among the wild larvae could have contributed to this behavioral difference we observed between laboratory and wild larvae. In wild populations, behavioral effects of NPV infection might not be as pronounced as those seen among the laboratory larvae. However, the results of the sampling tests with burlap and cadaver collections indicate that NPV infection causes altered vertical distribution of larvae in natural populations. These observations of released larvae were conducted on a single tree. Although behavior might be affected by tree structure, we have no reason to believe that the general pattern of behaviors observed was unique to the particular tree used in this study.

Cumulative NPV-caused mortality among reared larvae collected from under burlap bands was 12 to 33% lower than that observed among

**Fig. 4.** Comparison of cumulative NPV-caused mortality for three sampling methods (burlap collection, burlap counting, collection from all strata) in three sites (Otis 1, Otis 5, DPW).
reared larvae collected from all strata. In the same comparison, the cumulative proportions of NPV-killed larvae counted under burlap bands were about 3 to 30 times lower than cumulative NPV mortality among larvae collected from all strata. Aggregation in the upper canopy and less frequent utilization of burlap bands by infected larvae may explain these effects. We observed that infected larvae were found under burlap bands less frequently and tended to occur higher in the tree during the day compared with healthy larvae. Furthermore, the cadavers of NPV-killed larvae were found primarily at or near the tree top, rather than under burlap bands, which undoubtedly contributed to the very low estimate of disease prevalence obtained by cadaver counts of larvae under the burlap.

Although larval distribution is apparently affected by NPV infection, some released and wild infected larvae were found under burlap bands. It is likely that the behavior of infected larvae is not altered until the later stages of infection. Released, infected larvae were never observed in the topmost portions of the crown until after death. Thus, because the time of infection and mortality within a population is not synchronized among all individuals, a certain proportion of infected larvae will be in the early stages of infection at any particular time. Therefore, that proportion of infected larvae are likely to exhibit somewhat normal behavior, including resting diurnally under burlap bands.

Collection and rearing of larvae from under burlap bands may, therefore, provide a reasonable estimate of disease prevalence when a high degree of accuracy is not required. Although burlap sampling slightly underestimated the prevalence of the disease compared with more extensive sampling, the two estimates were roughly proportional.

Although much simpler than collecting and rearing larvae, counting cadavers under burlap bands may provide a less predictable estimate of NPV prevalence. Mortality estimated from cadaver counts differed from mortality estimated from larval rearings by a factor ranging from =3.2 to 8. Furthermore, mortality due to other pathogens, such as *Entomophthora maimaiga* Humbre, Shimazu, and Soper, or to some parasitoids, such as *Parasetigena sylvestris* (Robineau-Desvoidy), may not be easily distinguished from NPV-caused mortality without microscopic examination of the cadaver. In addition, the persistence or disappearance rate of cadavers under burlap bands may be influenced by factors, such as removal by scavengers, microbial degradation, or physical forces such as wind and rain, that could vary considerably among sites or years. However, the ease with which cadaver counts may be obtained may justify a sacrifice in accuracy for some applications. Therefore, this technique, as well as that of rearing larvae collected from under burlap bands, deserves to be more closely examined as viable alternatives to the labor-intensive, although undoubtedly more accurate, method of rearing larvae collected from all forest strata.

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