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# Studies on *Rhabdionvirus oryctes*

## I. Effect on Larvae of *Oryctes rhinoceros* and Inactivation of the Virus

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The mortality rates and the periods of lethal infection are described for the larvae of *Oryctes rhinoceros* inoculated with *Rhabdionvirus oryctes*. Ground-up virus-killed larvae of *O. rhinoceros* were used to prepare the inocula. Similar median lethal dosages were estimated for the different larval stages and for larvae kept under different conditions of food, temperature, and humidity after the inoculation. The period of lethal infection was found to be dependent on the larval stage and on the surrounding temperature. Storage of *Rhabdionvirus* in sawdust for 1 week reduced the amount of active virus material in the mixture to 0.091% of its initial value. After 2 weeks the amount of active virus material was reduced to 0.027%, and no more infectivity was detectable after 1 month. Drying the mixture or raising the temperature increased the rate of inactivation. The sterilization effect of heat and of several chemicals on the virus was studied.

### INTRODUCTION

*Rhabdionvirus oryctes* (ROV) was described by Huger (1966a) as a viral pathogen of the larvae of the Indian rhinoceros beetle, *Oryctes rhinoceros*. The virus was discovered in Malaya and was found to be absent in Western Samoa (Huger, 1966b). In 1967 the virus was introduced into Western Samoa as a control agent against *Oryctes rhinoceros* which is a serious pest of coconut palms in this country (Marschall, 1967). The disease became established readily (Huger, 1969a; Marschall, 1970) and is believed to have caused a significant reduction of the *O. rhinoceros* population (Marschall, 1970).

This successful introduction into Western Samoa stimulated detailed studies of the disease, especially the mode of transmission of ROV in the field and its effect on the *Oryctes* population. The first part of the studies described in this paper dealt with the mortality rate and the period of lethal infection in *Oryctes rhinoceros* larvae infected with ROV. By treating ROV preparations in various ways before the inoculation, it was also investigated how fast the virus is inactivated outside the host and how it can be sterilized effectively. Preliminary studies of these aspects were done by Huger (1966a, c, 1967).

### MATERIAL AND METHODS

*Beetles. Oryctes rhinoceros* adults were collected in 1970 in Viti Levu (Fiji Islands). The larvae were reared from eggs in the laboratory and were fed a mixture of decaying kapok wood and cowdung.

*Experimental methods.* The ROV used was obtained originally from infected *O. rhinoceros* larvae collected in the field in Western Samoa and subsequently propagated in the laboratory. The remains of ROV-killed third-instar larvae of *O. rhinoceros* were used as inocula. The inocula were prepared from larvae which had died recently and had shown the characteristic symptoms of the disease (see Huger, 1966a). The larvae were ground up in tap water (larvae:water, 1:4) with a blender operated at high speed for 2 minutes. The mixture was then filtered through a cloth. The resulting suspension containing ROV was stored in the deep freeze for up to 1 month before use.

Inoculation of the larvae with ROV was

performed in two different ways: (1) The ROV suspension was diluted with tap water to a given concentration and 0.05 ml of the diluted suspension was injected through the mouth into the esophagus of a paralyzed larva. The dosage was expressed in grams of ROV-killed larvae which were present in the 0.05 ml of the force-fed suspension. The control larvae were force fed with tap water only. After forced feeding, about 10% of the larvae regurgitated some gut fluid. Measurements showed that the amount of regurgitated fluid in general did not exceed half the amount of the force-fed fluid. (2) A second method of inoculation was by maintaining the larvae in mixtures of sawdust and ROV suspension. To prepare the mixtures, the sawdust was dried and the moisture was replaced by the diluted ROV suspension. As measurement for the concentration of ROV in the mixture, the percentage by weight of ROV-killed larvae in the mixture was given. The control larvae were maintained in an equivalent mixture of dry sawdust and tap water.

In the study of the inactivation of ROV under different environmental conditions, sawdust mixtures of different concentrations of ROV-killed larvae were stored for various lengths of time under different conditions of temperature before inoculation. In general, the mixtures were kept damp during storage, but in one experiment they were placed on trays in a layer 1 cm thick and were allowed to dry out at room temperature (average 26°C). The water lost during drying was replaced before the inoculation.

The effect of heat on ROV was studied by heating the suspension of ROV-killed larvae for 10 min at different temperatures before it was force fed to the larvae. The effect of chemicals on the virus was tested by mixing the ROV suspension with various concentrations of different chemicals 10 min before inoculation. Here control larvae were force fed with the equivalent concentration of the tested chemical. If an experiment was repeated, different ROV and larval material were used for each replicate.

The larvae were kept individually in tins covered with cloth. The tins contained either the ROV-sawdust mixtures or, in case of force-fed larvae, the normal larval food (kapok–cowdung mixture). Observations were made twice a week (daily in the case of first-instar larvae). The larvae were exposed by shaking the tins slightly; neither the medium nor the larvae were handled to reduce the risk of cross infection.

*Analysis.* The total mortalities 5 weeks after inoculation were corrected for mortalities in the control group by Abbott's formula. The median lethal dosages were determined by the maximum likelihood estimation (Finney, 1971). Since the mortalities varied in the different replicates, a separate $LD_{50}$ value was estimated for each replicate to give some indication of this variation. Averages and standard errors of the $LD_{50}$ values of the different replicates were based on the logarithms.

The $LD_{50}$ values were used to compare the susceptibility of the different larval stages to ROV. A measurement for the amount of active ROV remaining after a treatment of the inoculum was obtained by dividing the $LD_{50}$ value of the untreated inoculum by that of the treated inoculum. Here again, the averages from different replicates and the standard errors were based on the logarithms of these estimated values.

All dead control larvae and in many cases also the dead experimental larvae were bioassayed to establish the cause of their deaths. For a bioassay test, a freshly dead test larva was cut into small pieces and mixed with 10–100 g of kapok–cowdung medium, depending on the size of the larva. The mixture was fed to 2 healthy larvae held in one tin (experimental larvae) while keeping 2 more larvae as controls in a second tin. The result of this feeding test was called ROV-negative, i.e., the death of the original test larva was not caused by ROV, if both experimental larvae survived for 5 weeks or if 1 experimental larva died but the other survived its death for 5 more weeks. The result was called ROV-positive, i.e., the original test larva died from ROV, if both experimental larvae died in the first 5 weeks while the control larvae survived.

This criterion is sufficient since, except for *Metarrizium anisopliae*, which is easily recognizable, no other pathogen of *O. rhinoceros* has been found in Western Samoa (Huger, 1966b; Marschall, 1970). In a few cases, a doubtful test result was obtained when mortalities occurred among both the experimental and control larvae or when the experimental larvae died with atypical symptoms. In general, such tests were repeated with the dead experimental larvae as the new test larvae.

The periods of lethal infection were calculated from the time of death of only those larvae which gave a ROV-positive result in the feeding test. Deviations from the median periods of lethal infection were always expressed as standard errors.

## Results

On an average, 5% of the control larvae died in the first 5 weeks after inoculation. With one exception, none of these dead control larvae gave a ROV-positive result in the feeding test, thus indicating that the larval material used in the experiments was free of ROV-infections.

### *Mortalities and Periods of Lethal Infection in Larvae of* O. rhinoceros

Different concentrations of ROV in sawdust mixtures were tested with third-instar larvae. Twenty larvae were maintained in each concentration, and 3 replicates were conducted (average temperature 26°C). Table 3 (fresh mixture) includes the concentrations tested and the estimated $LD_{50}$ concentration in each replicate. The geometric mean of the $LD_{50}$ values of the three replicates was $2.8 \times 10^{-4}$% ROV-killed larvae. Combining all mortality data from the three replicates for a $LD_{50}$ estimation gave a similar mean $LD_{50}$ value of $2.9 \times 10^{-4}$% ROV-killed larvae in the mixture ($\chi^2 = 3.0$, 2 d.f.).

Forced feeding of third-instar larvae with different amounts of ROV ($10^{-8}$, $10^{-6}$, $10^{-4}$, and $10^{-2}$ g of ROV-killed larvae; 50 animals in each group and in the control group; average temperature 26°C) gave an estimated $LD_{50}$ value of $2.4 \times 10^{-4}$ g ROV-killed larvae force fed per larva ($\chi^2 = 5.1$, 2 d.f.).

In this experiment all larvae were kept until pupation. Except for the highest dosage, all dead larvae were bioassayed in feeding tests. Of all larvae dying from ROV; 94% did so during the first 5 weeks after inoculation and in no case later than 8 weeks (for average periods of lethal infection see below). Pupation of the surviving larvae took place at an average of 19 weeks after the inoculation, and the time of pupation was similar in all experimental groups and in the control group.

First-, second-, and third-instar larvae were kept in sawdust mixtures containing different concentrations of ROV ($10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$% ROV-killed larvae). In each of two replicates, 20 larvae were used per concentration and in the control group (average temperature 27°C). Table 1 gives the estimated $LD_{50}$ concentrations. Although the responses varied considerably between replicates, there was little difference between the $LD_{50}$ concentrations of the different instars in each replicate and between the average $LD_{50}$ values. On the other hand, the periods of lethal infection varied significantly ($P < 0.01$) for the

TABLE 1

Estimated, Median Lethal Concentrations of ROV[a] in Sawdust Mixtures, Given for Different Larval Stages of *Oryctes rhinoceros*[b]

| Larval instar | Test 1 | Test 2 | Geometric mean |
|---|---|---|---|
| First | $1.6 \times 10^{-3}$ | $2.4 \times 10^{-5}$ | $2.0 \times 10^{-4}$ |
| Second | $5.3 \times 10^{-4}$ | $7.3 \times 10^{-5}$ | $2.0 \times 10^{-4}$ |
| Third | $9.7 \times 10^{-4}$ | $9.7 \times 10^{-5}$ | $3.1 \times 10^{-4}$ |

[a] The concentrations are given in percentages (by weight) of ROV-killed larvae present in the mixtures.

[b] Each $LD_{50}$ value was estimated from mortalities with four different dosages (20 larvae/dosage). The mortalities were corrected for mortalities in the control group before the $LD_{50}$ estimation.

TABLE 2

MORTALITIES AND PERIODS OF LETHAL INFECTION IN THIRD INSTAR LARVAE OF *Oryctes rhinoceros* KEPT UNDER DIFFERENT CONDITIONS AFTER INOCULATION WITH ROV[a]

| | Treatment of larvae after inoculation | Mortalities of the larvae (%) | | | | Median periods of lethal infection in days |
|---|---|---|---|---|---|---|
| | | Controls | | Experiments | | |
| | | From ROV | From other causes | From ROV | From other causes | |
| Group 1 (Different food conditions) | Kept in sterilized sawdust | 0 | 10 | 25 | 5 | 22.0 ± 3.4 ($N$ = 5) |
| | Kept in kapok–cowdung mixture | 0 | 0 | 35 | 0 | 21.6 ± 3.7 ($N$ = 7) |
| Group 2 (different temperatures) | Kept at 25°C | 0 | 10 | 90 | 5 | 21.4 ± 3.0 ($N$ = 18) |
| | Kept at 27°C | 0 | 20 | 85 | 5 | 21.2 ± 2.1 ($N$ = 17) |
| | Kept at 32°C | 0 | 5 | 90 | 0 | 14.8 ± 1.7 ($N$ = 18) |
| Group 3 (different moisture conditions) | Kept in dry medium | 0 | 35 | 75 | 10 | 17.4 ± 1.1 ($N$ = 15) |
| | Kept in normal medium | 0 | 0 | 70 | 0 | 17.6 ± 1.2 ($N$ = 14) |
| | Kept in wet medium | 0 | 0 | 50 | 0 | 17.7 ± 0.8 ($N$ = 10) |

[a] Each percentage figure was obtained from 20 larvae. The experimental larvae were each force-fed with $10^{-4}$ g of ROV-killed larvae. The average temperature during the experiments of Groups 1 and 3 was 26°C.

three instars. In the mixture containing $10^{-4}$% ROV-killed larvae, the median periods of lethal infection in the second replicate were 8.5 ± 0.2 days ($N$ = 13) for the first, 12.6 ± 1.0 days ($N$ = 14) for the second, and 22.5 ± 2.8 days ($N$ = 10) for the third-instar larvae.

Twenty young third-instar larvae (2 weeks) and 40 old third-instar larvae (3 months) were force fed with $10^{-4}$ g ROV-killed larvae per animal (20 control larvae in each group, average temperature 27°C). No significant difference in mortalities from ROV occurred between both experimental groups (70 and 77%) but the period of lethal infection was significantly ($P$ = 0.002) shorter in the young third-instar larvae (17.6 ± 1.2 days, $N$ = 14) as compared to the old third-instar larvae (25.0 ± 1.3 days, $N$ = 31).

Three groups of third-instar larvae were force-fed with $10^{-4}$ g of ROV-killed larvae per animal and kept after inoculation under different conditions of food, temperature, or moisture. (Different ROV preparations and different larval material were used in each group.) Table 2 gives details of the experiment, the mortalities, and the periods of lethal infection. In all three groups the different conditions did not cause significantly different mortalities from ROV, but in the second group the higher temperature caused a significantly shorter period of lethal infection ($P$ = 0.03).

*Inactivation of ROV*

Sawdust mixtures were set up with different concentrations of ROV and stored for different lengths of times at ambient temperatures (average 26°C) before inoculation. Table 3 gives the estimated $LD_{50}$ concentrations of mixtures of different ages. From these $LD_{50}$ values the percentage of ROV (fresh material = 100%) remaining

TABLE 3

ESTIMATED, MEDIAN LETHAL CONCENTRATIONS OF ROV[a] IN SAWDUST MIXTURES OF DIFFERENT AGES[b]

| | Age of the mixtures at the time of the inoculation | | | |
|---|---|---|---|---|
| | Fresh | 1 Week | 2 Weeks | 1 Month |
| Test 1 | $2.3 \times 10^{-4}$ | 0.36 | 2.0 | No mortality |
| Test 2 | $9.7 \times 10^{-4}$ | 0.18 | 1.5 | No mortality |
| Test 3 | $9.7 \times 10^{-5}$ | 0.45 | — | — |
| Geometric mean | $2.8 \times 10^{-4}$[c] | 0.31 | 1.7 | (>10) |

[a] The concentrations are given as percentages (by weight) of ROV-killed larvae present in the mixtures.

[b] ROV-sawdust mixtures were stored for different lengths of time before the inoculation. Each $LD_{50}$ value was estimated from mortalities with different dosages after a correction for mortalities in the control group. The following initial concentrations were set up (in percentage of ROV-killed larvae) in fresh mixtures: $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$%, in mixtures stored for 1 or 2 weeks: 0.1% and 1%, and in mixtures stored for 1 month: 1% and 10%. In each replicate 20 third-instar larvae were kept per concentration, and for the determination of each $LD_{50}$ value, a control group of 20 larvae were kept.

[c] The standard error of this mean corresponds to the range $1.4 \times 10^{-4}$ to $5.4 \times 10^{-4}$% ROV-killed larvae.

active[1] after storage for 1 or 2 weeks was derived (Table 4).

The estimated $LD_{50}$ concentrations of ROV in sawdust mixtures stored for 1 week at different temperatures are given in Table 5. It was estimated from these data that in the tested temperature range, a 10°C higher storage temperature reduced the amount of active ROV present after 1 week to about one-tenth.

In a similar experiment, ROV-sawdust mixtures were kept dry or damp for 1 week. Besides the controls, the initial concentrations of 0.1% and 1% ROV-killed larvae were used in the test mixtures. Twenty

TABLE 4

ESTIMATED PERCENTAGES OF ROV REMAINING ACTIVE[a] AFTER STORAGE FOR ONE OR TWO WEEKS (FRESH MIXTURE = 100%)[b]

| | 1 Week | 2 Weeks |
|---|---|---|
| Test 1 | 0.034% | 0.011% |
| Test 2 | 0.54% | 0.065% |
| Test 3 | 0.022% | — |
| Geometric mean | 0.091%[c] | 0.027% |

[a] For explanation see footnote 1.

[b] The figures are derived from the $LD_{50}$ values of Table 3.

[c] The standard error of this mean corresponds to the range 0.035 to 0.24%.

TABLE 5

ESTIMATED, MEDIAN LETHAL CONCENTRATIONS OF ROV[a] IN SAWDUST MIXTURES AFTER STORAGE FOR ONE WEEK AT DIFFERENT TEMPERATURES

| Initial concentrations in the test mixtures[a] | Storage conditions before inoculation | Estimated $LD_{50}$ concentration[a, b] |
|---|---|---|
| $10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$% | Fresh | $9.7 \times 10^{-4}$% |
| $10^{-2}$, $10^{-1}$% | 1 week at 12°C | $1.1 \times 10^{-2}$% |
| 0.1, 1% | 1 week at 26°C | 0.18% |
| 1, 10% | 1 week at 32°C | 0.90% |

[a] The concentrations are given in percentages (by weight) of ROV-killed larvae present in the mixtures.

[b] Each $LD_{50}$ value was estimated from the mortalities with different dosages (see first column) after a correction for mortalities in the control group. Twenty third-instar larvae were kept per dosage and in each control group.

[1] The term "active ROV" is used in the sense that a given percentage of mortality is caused by a given concentration of "active ROV." For example, a fresh mixture with $2.8 \times 10^{-4}$% ROV-killed larvae and a 1-week-old mixture with an initial concentration of 0.31% ROV-killed larvae both caused 50% mortality, and thus both contained the same concentration of "active ROV." Since in this example $2.8 \times 10^{-4}$ is 0.09% of 0.31, 0.09% of the initial amount of ROV remained "active" after 1 week of storage (compare with Tables 3 and 4).

TABLE 6

MORTALITIES OF THIRD-INSTAR LARVAE FORCE FED WITH ROV SUSPENSIONS TREATED WITH HEAT OR CHEMICALS[a]

| Treatment of the ROV suspension | | Mortalities of the larvae (%) | |
|---|---|---|---|
| | | Controls | Experiments |
| No treatment, control for heat test | | 5 | 95 |
| 50°C for 10 min | | — | 15 |
| 60°C for 10 min | | — | 15 |
| 70°C for 10 min | | — | 0 |
| No treatment, control 1 for chemical test | | 0 | 90 |
| No treatment, control 2 for chemical test | | 5 | 95 |
| Containing 0.1% "Teepol" [b] | | 0 | 85 |
| Containing 10% ethyl alcohol | | 0 | 85 |
| Containing sodium hypochlorite[c] | 0.005% | 0 | 85 |
| | 0.05% | 0 | 35 |
| Containing formaldehyde | 0.1% | 0 | 80 |
| | 1% | 0 | 0 |
| Containing "Dettol" [d] | 0.1% | 0 | 85 |
| | 1% | 0 | 0 |

[a] Each percentage figure was obtained from 20 larvae. Each experimental larva was force fed with $10^{-2}$ g ROV-killed larvae and the control larvae with tap water or in the chemical tests with the chemical alone. The ROV suspensions and the chemicals were mixed 10 min before the mixtures were force-fed.

[b] Commercial detergent.

[c] Applied as "Clorox," a commercial bleach containing 5% sodium hypochlorite.

[d] Commercial germicide, containing 5% chloroxylenol.

third-instar larvae were kept per concentration per replicate; the average temperature was 26°C. After drying of the ROV-mixture, no mortality occurred in the first replicate and 5% in each concentration of the second replicate. The mixtures that were kept damp gave the normal responses (estimated average $LD_{50}$ concentration 0.40% ROV-killed larvae in the mixture).

Table 6 gives the mortalities obtained by forced feeding of the larvae with ROV suspensions treated with heat or chemicals. ROV was inactivated most effectively by applying 70°C for 10 min and by a 1% solution of formaldehyde or "Dettol" (a commercial germicide).

## DISCUSSION

The results described above suggest that the mortality of *O. rhinoceros* larvae from ROV is not influenced significantly by the different developmental stages of the larvae or by different environmental conditions after inoculation. On the other hand, the period of lethal infection depends greatly on the developmental stage of the larvae and is also affected by the surrounding temperature. The observation that the period of lethal infection is restricted to 8 weeks (pupation took place after 19 weeks) suggests that either ROV infections develop immediately after inoculation or the larvae remain uninfected. No evidence was obtained for so called sublethal infections which could cause delayed outbreaks of the disease.

The results indicate that ROV is inactivated rapidly at ambient temperature, thus confirming the earlier work by Huger (1966c, 1967). It can be concluded that ROV-material, even at the highest concentrations, after storage at ambient temperature for more than 2 weeks, is not able to infect a significant percentage of the larvae. Dry and hot conditions increase the inactivation rate. These results suggest that ROV material in the form of macerated ROV-killed *O. rhinoceros* larvae is unsuitable for field release.

The results also show that the inactivation rate is higher in the first week compared with that of the second week of storage. This could mean that some of the ROV particles in the mixture were more resistant to denaturation than others.

For sterilizing ROV, heat seems to be most effective. The sterilization experiments with chemicals gave only comparative information for the different chemicals tested. No accurate sterilization time for a given concentration of a chemical can be stated from these as the chemical and the ROV could have still reacted with each other in the gut of the larvae after forced feeding.

In the present experiments the inocula presumably contained ROV particles in various forms. In his initial studies, Huger (1966a) described two forms of ROV particles, spherical and rod-shaped. He regarded the spherical particles as early developmental stages, and the rods as mature forms. Recent studies by Huger (1969b, 1970) have revealed that the rod-shaped particles are sometimes associated with inclusion bodies. The inclusion bodies, however, are restricted to a small area of the midgut epithelium and, moreover, can not be demonstrated in every larva infected with ROV. No statement about the proportions of these different particles in the inocula used in the present experiments can be made, although the percentage of ROV particles in inclusion bodies is apparently low.

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