



**AgEcon** SEARCH  
RESEARCH IN AGRICULTURAL & APPLIED ECONOMICS

*The World's Largest Open Access Agricultural & Applied Economics Digital Library*

**This document is discoverable and free to researchers across the globe due to the work of AgEcon Search.**

**Help ensure our sustainability.**

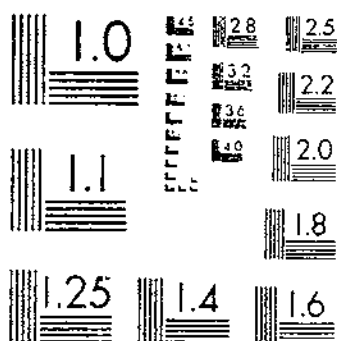
Give to AgEcon Search

AgEcon Search  
<http://ageconsearch.umn.edu>  
[aesearch@umn.edu](mailto:aesearch@umn.edu)

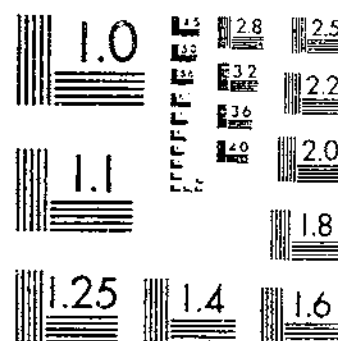
*Papers downloaded from **AgEcon Search** may be used for non-commercial purposes and personal study only. No other use, including posting to another Internet site, is permitted without permission from the copyright owner (not AgEcon Search), or as allowed under the provisions of Fair Use, U.S. Copyright Act, Title 17 U.S.C.*

1 TB 1528 (1976) USDA TECHNICAL BULLETINS 1528 UPDATA  
2 BIOASSAY OF BACILLUS THURINGIENSIS (BERLINER) ENDOTOXIN USING THE  
3 DULNAGE, H. T. MARTINEZ, A. J. PENA, T. J. 1 OF 1

# START



MICROCOPY RESOLUTION TEST CHART  
NATIONAL BUREAU OF STANDARDS-1963-A



MICROCOPY RESOLUTION TEST CHART  
NATIONAL BUREAU OF STANDARDS-1963-A

REFERENCE  
DO NOT LOAN

**BIOASSAY OF *BACILLUS*  
*THURINGIENSIS* (BERLINER)  
 $\delta$ -ENDOTOXIN  
USING THE TOBACCO BUDWORM**

Technical Bulletin No. 1528

U. S. DEPOSITORY

JUN 01 1955

Los Angeles Public Library

Agricultural Research Service  
UNITED STATES DEPARTMENT OF AGRICULTURE  
in cooperation with  
Texas Agricultural Experiment Station

## ACKNOWLEDGMENTS

The authors wish to thank H. T. Dulmage, Jr., who donated his time and skill to take and process the photographs used herein. We would also like to thank the many Neighborhood Youth Corps and Operation Mainstream workers who assisted in these assays, and who, through their interest and their willingness to offer suggestions, contributed much to the efficiency of the mechanics of the assay.

Washington, D.C.

Issued May 1976

# CONTENTS

	Page
Acknowledgments .....	ii
Abstract .....	1
Introduction .....	1
Assay procedures .....	3
Diet preparation .....	3
Agar solution .....	4
Diet ingredients .....	4
Cup preparation .....	4
Samples and dilutions .....	4
Controls .....	7
Infestation .....	7
Reading the test .....	10
Computing potency in international units .....	10
Assay accuracy .....	11
Replication .....	11
Reproducibility .....	12
Manpower requirements .....	12
Conclusion .....	14
Literature cited .....	14

## ILLUSTRATIONS

Fig.	
1. Trays being set up with cups for day's assays .....	5
2. Blenders during assay .....	6
3. Diet being dispensed into cups on trays .....	8
4. Infesting the assay .....	9
5. A 7-day-old larva retarded by exposure to the <i>B. thuringiensis</i> $\delta$ -endotoxin compared with a normal larva of the same age .....	9

## TABLES

1. Distribution of coefficients of variation determined in replicate assays of various formulations of the $\delta$ -endotoxin produced by <i>B. thuringiensis</i> .....	12
2. Reproducibility of the bioassay procedures used at Brownsville, Tex., to measure the potency of formulations of the <i>B. thuringiensis</i> $\delta$ -endotoxin; comparison of bioassay results testing the same samples over a period of 6 years .....	13

Trade names are used in this publication solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over other products not mentioned.

# BIOASSAY OF *BACILLUS THURINGIENSIS* (BERLINER) $\delta$ -ENDOTOXIN USING THE TOBACCO BUDWORM

By H. T. DULMAGE, microbiologist, A. J. MARTINEZ,<sup>1</sup> agricultural research technician, and T. PEÑA, biological technician, Cotton Insects Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Brownsville, Tex. 78520

## ABSTRACT

This bulletin presents in detail the mechanics of a bioassay developed to measure the potencies of formulations of *Bacillus thuringiensis* (Berliner)  $\delta$ -endotoxin, using the tobacco budworm, *Heliothis virescens* (F.). No new assay principles are introduced, but the techniques described have improved the efficiency of the assay and are fast, convenient, and reliable. The methods have been used successfully for several years, and data on the accuracy and reproducibility of the assay are presented. The techniques are readily transferable to assays against other insects and with other insecticidal materials. KEY WORDS: *Heliothis*, *Heliothis virescens*, Tobacco budworm, HD—1, Bioassay, *Bacillus thuringiensis*,  $\delta$ -endotoxin.

## INTRODUCTION

Formulations of the  $\delta$ -endotoxin produced by *Bacillus thuringiensis* (Berliner) are used in the control of several lepidopterous pests. However, if these materials are to enjoy still wider use, they must be made more effective and less expensive: i.e., we must increase the insecticidal activity of these products and re-

duce the cost of producing them. To accomplish this, we will first need an accurate means of measuring the active ingredient. The method we choose must be accurate yet sufficiently simple to enable us to run samples for our research at a rate which will insure reasonable progress. Because chemical assays are both accurate and rapid, it would be helpful if there were a generally accepted chemical assay for the  $\delta$ -endotoxin. Unfortunately, there is

<sup>1</sup>Present address: Western Cotton Insects Laboratory, 4207 East Broadway Rd., Phoenix, Ariz. 85040.

none, and at present, we have no way of measuring the quantity of  $\delta$ -endotoxin in a formulation except through a bioassay.

Insect bioassays are difficult to conduct, and early workers tried to avoid them. Since microscopic examination of *B. thuringiensis* cultures appeared to show one crystal of the  $\delta$ -endotoxin for every spore of the bacillus, it was hoped that a count of viable spores present in a preparation could be used to determine its insecticidal activity. The spore count became a generally accepted method of measuring the potencies of *B. thuringiensis* formulations.

Bonnefoi et al. (1)<sup>2</sup> first warned against the use of the spore count and pointed out the need for a bioassay. A year later, Burgerjon (2) further noted that an accurate bioassay required that the response of insects to a test sample be compared with their response to a reference standard. To make his comparisons, Burgerjon measured the degree of feeding inhibition in *Pieris brassicae* after exposure to the  $\delta$ -endotoxin. Splittstoesser and McEwen (15) suggested that bioassays of  $\delta$ -endotoxin could be improved if the toxin were administered to test insects by incorporating it into an artificial diet. They used death as the assay criterion, calculating the  $LC_{50}$  of their samples from the response of the insects to a series of dilutions of the toxin in the diet. Later, Mechalias and Anderson (12) reviewed the use of a standard in an

assay proposed by Mechalias and Dunn (13) and emphasized that the regression curves used to compute the  $LC_{50}$ 's of the standard and the test samples should be considered parallel.

In spite of these and other studies, the spore count continued to be used to standardize formulations of *B. thuringiensis*, and bioassays were used only infrequently. Then in 1966, in Wageningen, the Netherlands, participants in a symposium on the standardization of microbial insect control agents proposed that a formulation of the *B. thuringiensis*- $\delta$ -endotoxin-spore complex prepared for the Institut Pasteur, Paris, France, and named E-61, be adopted as a primary international reference standard. The same group assigned a potency of 1,000 international units (IU)/mg to E-61 and further recommended that the potency of all preparations of the  $\delta$ -endotoxin be directly or indirectly compared to E-61 and be expressed in IU's. Burges (3) has reported the details of this meeting.

Although the concept of expressing potencies in IU's was a much needed advance, it was not generally accepted until the discovery of the HD-1 isolate of *B. thuringiensis* by Dulmage (6). Formulations of this isolate, although no higher in spore count than previous preparations of the  $\delta$ -endotoxin, were many times more potent, and this improved potency was demonstrated both in the laboratory and in field trials. Thus, the spore count was obviously not valid, and the need for a bioassay was demonstrated.

<sup>2</sup>Italic numbers in parentheses refer to items in "Literature Cited," p. 14.

Dulmage et al. (9) next proposed a bioassay based on 4-day old larvae of the cabbage looper, *Trichoplusia ni* (Hübner). This assay was adopted by the Pesticides Regulation Division of the Environmental Protection Agency as the official bioassay for use in measuring the potencies of formulations of *B. thuringiensis* offered for sale in the United States. Later, as reported by Dulmage (8), a formulation of HD-1, labeled HD-1-S-1971, was adopted as the primary reference standard for use in this assay and assigned a potency of 18,000 IU/mg.

This last assay was very satisfactory, but was not meant to preclude the use of other assay procedures, particularly in evaluating formulations of *B. thuringiensis* against specific target insects, since, as Burges (4) has pointed out, several questions as to whether the endotoxins produced by different strains of *B. thuringiensis* have the same spectrum of potency remain unresolved. Dulmage (7) has discussed some aspects of this problem.

The tobacco budworm, *Heliothis virescens* (F.), is a major pest of tobacco and cotton. McGarr et al. (10, 11) have demonstrated that formulations of HD-1 will control *Heliothis* species on cotton, but only when applied at rates that are presently impractical. In the course of searching for means of producing more potent and less expensive formulations of  $\delta$ -endotoxin for use in the control of *Heliothis*, we developed an assay based on neonate larvae of the tobacco budworm. The mechanics developed for this assay make it both

rapid and accurate, and we believe that a detailed description of our methods will be useful to other workers, since many of our procedures can readily be adapted for use in assays with other microbial insect control agents or against other insects. This bulletin describes our assay.

## ASSAY PROCEDURES

In this assay, as in the procedure proposed by Dulmage et al. (9), serial dilutions of the test samples and of a standard preparation of known potency are incorporated into an artificial diet. The diet-sample mixtures are then distributed into  $\frac{3}{4}$ -oz clear plastic cups and infested with one neonate larva of the tobacco budworm per cup. After 7 days, the cups are examined, and the percentage of kill for each dilution is recorded and used to calculate the  $LC_{50}$  in each sample. The potencies of the samples are then determined by comparing the  $LC_{50}$  of individual samples with that of the standard.

It is essential for accuracy that any insect used in an assay be vigorous and disease free. The culture of the tobacco budworm being reared by the mass-rearing group at our laboratory meets these standards, and we routinely obtain newly hatched, treated eggs from them. The treatment and handling of the eggs will be discussed in a later section.

## Diet Preparation

Proper preparation of the diet is essential to an accurate assay, since the thick, lumpy diet which comes from poor preparation makes it dif-

ficult to get even distribution of the sample in the diet. The key to good diet is in taking care that the agar has been completely dissolved. To make 1 liter of diet, the agar solution and diet are prepared and mixed as described below.

### Agar solution

Bring 270 ml  $H_2O$  to a vigorous boil. In a separate container, blend together 15 g agar and 230 ml cold  $H_2O$ . Add the blend to the boiling  $H_2O$ , heat the mixture again to boiling, and boil for 2 min. Yield: 500 ml agar solution.

### Diet ingredients

To 500 ml cold  $H_2O$ , add, while stirring, 6 ml 4 N KOH. Continue to stir and add the following ingredients: 146.21 g nutrient mix,<sup>3</sup> 4.83 g ascorbic acid, and 0.17 g chlortetracycline  $\cdot$  HCl.<sup>4</sup> Then add, while continuing to stir: 5.0 ml 10% formaldehyde solution, 13.3 ml 15% choline chloride solution, and 4.0 ml vitamin solution.<sup>5</sup>

<sup>3</sup>Composition of nutrient mix, grams per kilogram: Soybean flour, 551.7; wheat germ, 246.2; Wesson salts mixture, 82.07; sucrose, 100.3; methyl parabenzoate, 12.31; sorbic acid, 7.41. (All ingredients for 1 week's assays are weighed at the same time and stored in plastic bags at room temperature until needed.)

<sup>4</sup>As pure antibiotic. Our sources have varied. At present, we are using 3.08 g of a feed-grade formulation containing 25 g chlortetracycline HCl/lb.

<sup>5</sup>Composition of vitamin solution, grams per liter: calcium pantothenate, 12.0; nicotinamide, 6.0; riboflavin, 3.0; folic acid, 3.0; thiamine  $\cdot$  HCl, 1.5; pyridoxine  $\cdot$  HCl, 1.5; biotin, 0.12; vitamin  $B_{12}$ , 0.006. The mixture is held in the refrigerator until used. A fresh solution is prepared every 2 weeks.

Mix the diet ingredients thoroughly, then add the hot agar solution, continuing to stir vigorously. Final temperature: 53°–55° C. The diet is kept constantly stirred in a mixing tank until used. A plastic bag is kept over the tank and mixer to prevent contamination of the diet. The mixing tank is cleaned thoroughly each day after use and is autoclaved at 121° C for 35 min under steam at least once a week as a further precaution against any buildup of contamination within the tank.

### Cup Preparation

When maximum accuracy is desired, we use 50 cups per dilution of sample. For routine or preliminary assays, we use 25 cups. From  $\frac{1}{4}$ -in plywood and quarter round, we constructed a set of trays with inside dimensions of 8 by 16 in, each of which holds 50 clear plastic  $\frac{3}{4}$ -oz cups. These trays are coated with varnish that allows them to be washed with water and antiseptic. (The trays are washed daily; they can be steam-sterilized and are autoclaved at least once a week.) Each tray thus holds enough cups to accommodate either one or two dilutions of a sample depending upon the procedure used. The necessary number of trays is set up with cups early in the morning while the diet is being made, rather than on the day before, to avoid possible airborne contamination. The trays are shown in figure 1.

### Samples and Dilutions

The initial suspensions and all dilutions of the test samples and the



PS-4400

FIGURE 1. — Trays being set up with cups for day's assays.

standard are made in a buffered saline solution consisting of the following ingredients (grams per liter): NaCl, 8.5;  $K_2HPO_4$ , 6.0; and  $KH_2PO_4$ , 3.0. When this solution is used to make the initial suspension, 1.0 ml of a 1% Tween 80 solution is added to each 100 ml of the saline solution to aid in wetting the test sample. The factor at which the samples are diluted in the diet is chosen for convenience and does not affect the accuracy of the assay. We dilute samples at either 1:10 or 1:50 in the diet; however, the dilution factor is kept constant within a day's assays. Since in the series of dilutions we use, we want the highest concentration of sample in the diet to be about 3 times the  $LC_{50}$  of the sample, an appropriate initial concentration of the test material is approximately 30 times the estimated  $LC_{50}$  when a 1:10 dilution is to be used and 150 times the estimated

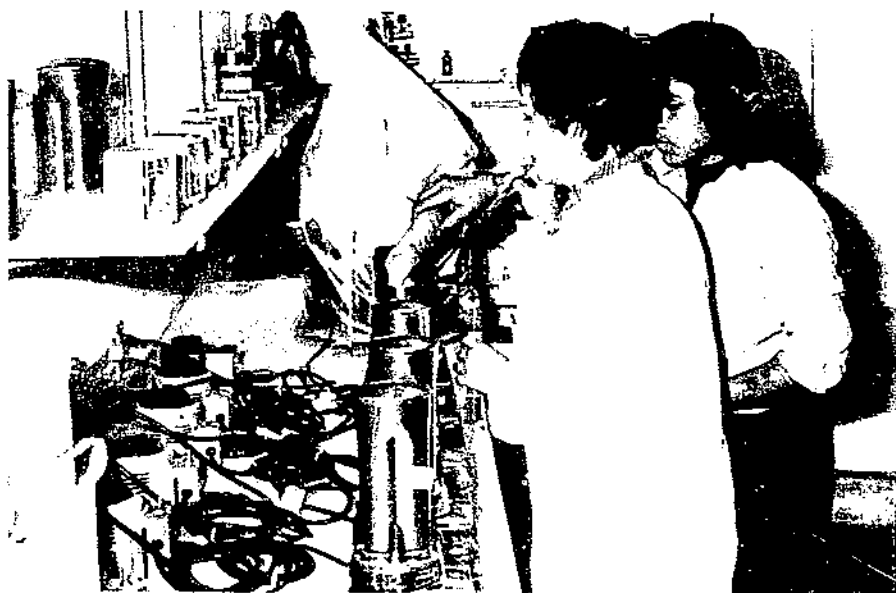
$LC_{50}$  when a 1:50 dilution is used. In the past, we sonified the initial suspensions of each sample to aid in the complete dispersion of the toxin, but we have since found it quicker and more convenient to homogenize the suspension briefly by hand in a 50- or 100-ml Ten Broeck tissue grinder for about 30 s.

Seven dilutions are usually tested in an assay. Since the highest accuracy is obtained when the points are clustered as closely as possible about the  $LC_{50}$ , we make the following series of dilutions from the initial suspensions: 3:4, 1:2, 3:8, 1:4, 3:16, and 1:8. These dilutions are derived from a series of 1:2 dilutions from both the undiluted and the 3:4-diluted sample suspensions. The 1:2 dilutions are conveniently made by mixing equal volumes of sample and saline solution in a 50-ml screw-top test tube. The tubes are labeled with masking tape, and the concen-

tration listed on the tape as it will be when the suspension is diluted in the diet. (The use of masking tape is important in the mechanics of our assay, as will be explained later.) Each suspension is agitated with a vortex test-tube mixer just before making the next dilution from it and again just before adding it to the diet. All dilutions for the day are prepared in advance of the assay, but none is ever held for more than 2 or 3 h.

When we are ready to incorporate the saline suspensions into the diet, we draw an appropriate amount of diet from the mixing tank and simultaneously pour the diet and pipet a proportional volume of one of the dilutions into a Waring Blendor jar (fig. 2). (When 50 cups are to be

used, we blend 20 ml suspension into 180 ml diet if we are using a 1:10 dilution in the diet, or 4 ml suspension into 200 ml diet for a 1:50 dilution; when 25 cups are used, we blend 10 ml suspension and 90 ml diet for the 1:10 dilution or 2 ml suspension and 100 ml diet for 1:50.) In most of our work, we have used the 1,000-ml blender jar, but recently have found that the 500-ml jar is more satisfactory and now use the smaller size. The mixture is then blended 2 min at high speed while the masking tape label is transferred from the test tube to the blender jar. (The masking tape label is transferred from step to step throughout the assay to minimize human error.) Since each sample must blend 2 min, we overlap blendings at 30-s inter-



PN-4401

FIGURE 2. -- Blenders during assay. Note the masking tape labels on the blenders, the timers on the shelf, and the variable transformers against the wall. The mixing tank in the background is kept covered to protect the diet from airborne contamination.

vals, using timers for each sample as shown in the background of figure 2. Theoretically, only four blenders are needed to maintain such a sequence; however, we find the blender motors last longer if they are allowed to cool between use, so we use six bases during the operation—only four of which are in use at any given time. Blenders start suddenly at high speed, resulting in considerable splash within the jar. Because this might lead to inaccurate blending, we leave the base speed on high but gradually bring the blenders up to speed with the variable transformers seen in the background of figure 2. We have learned that in the next step an experienced person can take the diet from a blender jar and fill 50 cups in about 15 s, so it is feasible, if desired, to increase the number of samples run at a time by decreasing the time interval between blendings.

After the diet and the sample have blended 2 min, the blender jar is removed, and the diet is transferred to a plastic mustard dispenser. The lid is put on, the dispenser inverted, and the diet dispensed into each of 25 or 50 cups by simply squeezing the sides of the dispenser. Since the assay depends on *concentration* and not on *quantity* of toxin, it is not necessary to accurately control the amount of diet per cup. The masking tape from the blender jar is transferred to the side of the tray containing the cups. After they are empty, the jars and dispensers are washed and reused. As far as our tests show, no cross-contamination between samples has resulted from

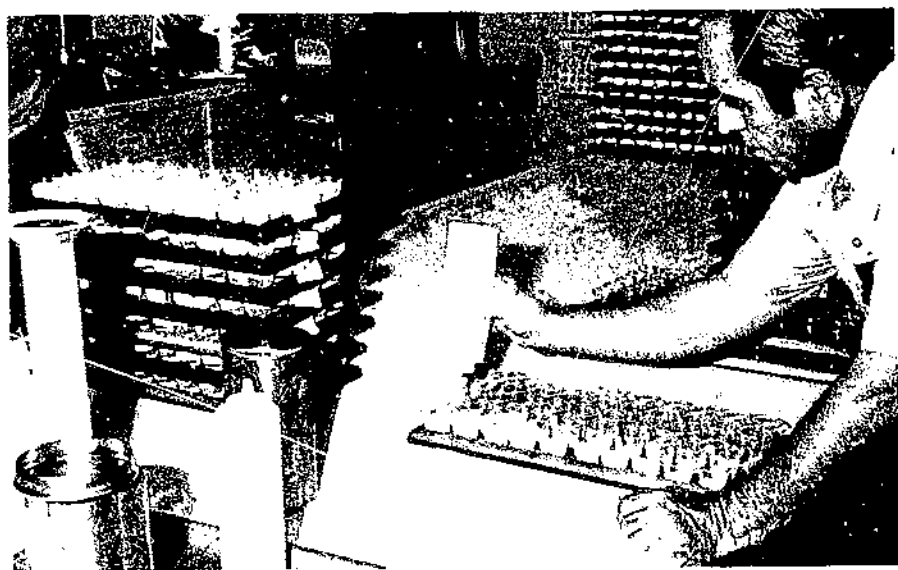
this reuse. However, all glassware is thoroughly washed and autoclaved at the end of each day as a precaution against any buildup of contaminating material. Figure 3 shows the dispensing of the diet.

### Controls

Control samples, using buffer only as an additive to the diet, are tested in every assay—one at the beginning and one at the end of the assay. No corrections in the final calculations are made for control deaths, but any assay with more than 4% deaths in either of the control samples is considered very suspect.

### Infestation

*Larvae*.—We get eggs from the mass-rearing facility 2 days in advance of an assay. These eggs have previously been treated for 2 min with a 0.2% solution of sodium hypochlorite, washed with water, and air-dried in a laminar-flow hood. (For details of the rearing procedures and egg treatment, see Raulston and Lingren 14.) To insure an abundant supply of newly hatched larvae at the time we need them, we usually obtain a large excess of eggs—approximately 1 ml of eggs for each seven trays. (One ml contains about 7,000 eggs.) The eggs are then distributed in 9-oz waxed paper cups in quantities sufficient to lightly cover the bottom of each cup. No attempt is made to measure the volume of eggs placed in each cup, but it averages about three-fourths milliliter per cup. A single Kimwipe is loosely crumpled and placed in each cup. The cups are then covered



PN-402

FIGURE 3.—Diet being dispensed into cups on trays. Completed trays labeled with masking tape are in the left background.

with a translucent plastic lid and placed in an incubator in the dark at  $30^{\circ} \pm 1^{\circ} \text{C}$  and 49%–50% relative humidity for a period of approximately 44 h, at the end of which time the larvae should begin hatching. The exact incubation period must be determined by trial and error, but should be timed so that hatch starts 4 to 5 h before infestation is to begin.

*Infesting.*—The diet in the cups is allowed to harden for a minimum of 45 min (usually over the lunch hour), and each cup is then infested with one neonate larva of the tobacco budworm, using the procedure described below.

About one-half hour before we begin infesting the assay, the cups containing the eggs are brought out to the light so that the more vigorous larvae will climb up to the underside of the translucent lid. Larvae for use

in the assay are obtained by removing a lid and tapping it gently over the white plastic table covering we use for infesting. Larvae that fall from the lid to the table are readily visible on the white top. The lid is replaced on the egg cup to collect more larvae. The assay cups are then infested with the neonate larvae, using a fine camel's hair brush, taking care to see that the brush does not touch the diet. If the brush touches, it is discarded. A different brush is used for each dilution to make sure that there is no carryover of toxic material from one sample to another, and, at the end of each day, all brushes are placed in a glass container and steam-sterilized for use the following day. While one worker puts the larvae in the cups, another caps the cups, using plastic-coated lids so that the diet will not dry out



PN-4403

FIGURE 4.—Infesting the assay. Note the sterilized camel's hair brushes in the flask in the foreground, the white table top, and, in the background, the completed samples in the bags with masking tape labels.



PN-4404

FIGURE 5.—A 7-day-old larva retarded by exposure to the *B. thuringiensis*  $\delta$ -endotoxin compared with a normal larva of the same age.

during the incubation period. The capped cups from each dilution are placed in separate paper bags, the masking tape label from the corresponding tray is transferred to each bag, and the bag is stored in an incubator at  $30^{\circ} \pm 1^{\circ} \text{ C}$  and 49%–50% relative humidity. Figure 4 shows workers infesting the assay.

### Reading the Test

After 7 days of incubation the cups are removed from the incubator, and the number of living and dead larvae in each dilution is determined. When it is uncertain whether a larva is alive or dead, it is touched with the point of a needle or pencil. No consideration is given to imminent death; if there is any response, no matter how feeble, the larva is recorded as alive. Usually, however, most deaths occur in the first stage, and dead larvae are easily recognizable. In such cases, the cups need not be opened, since the clear plastic gives an adequate view of the larvae. It must be stressed that death is the only criterion. Many larvae will be obviously damaged and severely retarded by exposure to the toxin. Figure 5 shows a severely stunted but still living 7-day-old larva that has been exposed to *B. thuringiensis*  $\delta$ -endotoxin compared with a normal larva of the same age. Such damage is ignored for the purposes of the assay.

### Computing Potency in International Units

The  $LC_{50}$  of each sample is computed from the data obtained on the percentage of kill in each of the

series of dilutions of the sample. The potency of the sample is then calculated by the following formula:

$$\text{Potency sample (IU/mg)} = \frac{LC_{50} \text{ standard} \times \text{potency standard (IU/mg)}}{LC_{50} \text{ sample}}$$

The  $LC_{50}$  can be computed in any one of the three ways described in the following paragraphs.

*Potency probit analysis.*—Daum (5) devised a potency probit analysis program for computers that computes the slopes of the regression curves of all samples within a day's assays; compares the slopes for parallelism, rejecting samples with nonparallel slopes; adjusts the curves of the acceptable samples to the average slope for the day; calculates the  $LC_{50}$  of each sample based on the corrected average slope; and then computes the ratio,  $LC_{50} \text{ sample} \div LC_{50} \text{ standard}$ , giving the ratio and the 95% confidence limits surrounding it. The program above has been modified by H. Del Var Peterson of the Biometrical Services Staff, Agricultural Research Service, College Station, Tex., to carry out the complete computation of potencies, so that the computer gives, in its printout for each sample, the slope of the regression curve, the  $LC_{50}$ , the ratio,  $LC_{50} \text{ standard} \div LC_{50} \text{ sample}$  (the inverse of Daum's program), and the potency in IU's with its 95% confidence limits. This method gives the most accurate results and should be used whenever a biometrician is available.

*Probit analysis.*—Frequently, advanced calculators can be used to run a probit analysis of each sample,

determining an  $LC_{50}$  for each sample within 95% confidence limits. Programs are available for some programmable calculators that can do this automatically. With this method, curves are not compared or corrected for parallelism, but good judgment on the part of the scientist can compensate for these shortcomings, and though the method is not as accurate as a potency probit analysis, it can yield good results. Of course, the experimenter himself must complete the calculations for 11's from the  $LC_{50}$  determinations.

*Log-probability paper.*—The percentage of kill for each dilution of a sample can be plotted on log-probability paper, a straight-line curve fitted to these points by eye, and the  $LC_{50}$  of the sample estimated from this curve. This procedure is adapted from a paper by Meehalas and Anderson (12), and works best for us when we use the data from several day's assays to determine a "typical" slope and then assume that the slope is constant from sample to sample and from day to day. We cut a clear plastic triangle to the slope of this typical curve and use the triangle to fit a curve to the points of each assay. When the location of the points is such that the curve will not fit them well, we reject the assay. There is one danger in the use of this procedure; we have found that the slope of the "typical curve" can change (why we do not know), although it does remain constant over periods of weeks or months. When such changes occur, they persist, also over long periods of time. Therefore, we reevaluate our "typi-

cal slope" frequently and adjust our triangle to fit any changes that may have occurred. The results obtained from these graphs are not as accurate as those obtained from mathematical analyses, but they are accurate enough to satisfy many of the needs of our research.

### ASSAY ACCURACY Replication

The assay should be replicated on at least 3 separate days for any critical determination. If the data from the replicates are pooled before being used to calculate the potencies, statistical analysis of the results will show the maximum precision. However, we feel that this precision is often illusory and thus prefer to calculate the potencies of each day's replicate and then determine the precision of the average of these individual determinations. Precision is influenced in three ways: by the variation inherent in the assay itself, by the homogeneity of the insect population used, and by the homogeneity of the sample—a nonhomogeneous sample will give widely varying results in an assay. Dulmage (7) has discussed the coefficients of variation observed between individual assays of 77 samples when our assay procedure was used and the potencies computed by potency probit analysis. His data are presented in table 1. The higher coefficients appeared to be associated with the sample and reflected poor homogeneity. Dulmage concluded that the coefficient of variation inherent in the assay was less than 0.15 and

TABLE 1.—*Distribution of coefficients of variation determined in replicate assays of various formulations of the  $\delta$ -endotoxin produced by B. thuringiensis*<sup>1</sup>

Range of coefficients of variation	Samples in range
< 0.050	3
0.050-0.099	12
.100-.149	20
.150-.199	18
.200-.249	15
.250-.299	7
> 0.300	2

<sup>1</sup>From Dulmage (7). Assay insect, *H. virescens* 77 samples.

greater than 0.12. Thus, three replicates are needed to determine the potency of a preparation within an error of 20% at a 90% confidence level, or four replicates at a 95% confidence level.

### Reproducibility

There is always a danger in any bioassay that some unrecognized variation—perhaps in the bioassay procedures, perhaps in the test insect—can change the assay results. If this variation is consistent, the accuracy of the assay may appear unchanged. Van der Geest and Wassink (16) compared different assay methods and showed that the potencies determined could be affected by the procedure used. It is therefore important in any bioassay system to check assay results at intervals to see if there has been any change. Fortunately, dry formulations of the *B. thuringiensis*  $\delta$ -endotoxin are very stable, so the assay may be checked by retesting samples from previous years and

comparing present determinations with those obtained earlier. Several months often elapse between assays on substances in which we have only moderate interest, and we use the reassays on these materials to some extent to monitor the reproducibility of our assay. However, during 1973, we had occasion to reassay several samples which had previously been assayed as early as 1968. Selected examples of these assays are presented in table 2. The data show that our measurement of potencies has remained very consistent over the years.

There were three samples, not included in table 2, whose 1973 assays did not correlate well with earlier assays. In each case the 1973 result deviated by 50%–100% from the earlier determinations: two were higher, one was lower. When we reexamined the earlier data, we found some cause to suspect that the earlier assays were in error, but there is no way now to prove it. None of these formulations were from the HD-1 fermentation, and there is always the possibility that the discrepancies observed indicate a lack of homology between these formulations and the standard, which was derived from HD-1. As Burges (4) has pointed out, it is impossible to compare two materials in a bioassay unless the active principles are homologous.

### MANPOWER REQUIREMENTS

Our goal is to make between 14 and 15 assays per day, including the standard. This assumes 7 dilutions for each sample, plus 2 controls for

TABLE 2.—*Reproducibility of the bioassay procedures used at Brownsville, Tex., to measure the potency of formulations of the B. thuringiensis  $\delta$ -endotoxin: comparison of bioassay results testing the same samples over a period of 6 years*

Culture number	Sample number	Average potency (IU/mg) during <sup>1</sup> —						Overall average potency (IU/mg)	Coefficient of variation
		1968	1969	1970	1971	1972	1973		
HD-1	RX-71-3	11,600(1)	13,700(1)	.....	.....	10,700(2)	13,100(3)	12,300	0.11
HD-1	RX-72-1	.....	13,200(1)	.....	.....	18,890(2)	16,400(3)	17,200	.17
HD-1	Comm. 152	.....	10,400(2)	.....	.....	.....	11,700(2)	11,000	.11
HD-1	Comm. 212	.....	10,400(7)	11,300(7)	11,100(1)	.....	10,700(3)	10,800	.15
HD-1	Comm. 1968	.....	.....	11,300(4)	.....	.....	9,680(2)	10,800	.20
HD-73	R-282	.....	.....	17,800(2)	.....	.....	18,800(3)	18,400	.08
HD-73	R-309	.....	.....	59,900(3)	.....	.....	68,400(2)	63,300	.18
HD-78	R-139	.....	62,100(1)	63,700(3)	.....	.....	62,100(3)	62,800	.16
HD-78	R-284	.....	.....	44,100(8)	.....	.....	47,000(2)	44,600	.17
HD-187	R-392C	.....	.....	.....	59,700(2)	.....	52,500(2)	56,100	.10
HD-187	R-418C	.....	.....	.....	.....	85,600(5)	63,700(2)	79,300	.20

<sup>1</sup>Number in parentheses after potency indicates the number of determinations on which the potency is based.

the day's assays, and 50 larvae for each dilution. To accomplish this, we have employed several combinations of workers. We have used one full-time technician and five part-time, unskilled workers, many of them high school students, who have proved to be very satisfactory. At present, we are using one full-time technician, two full-time, unskilled workers, and one part-time student. In either case, the day can be divided as follows: One unskilled worker spends about 2 h setting up the trays and getting the diet ready, while the technician and another worker spend about the same amount of time weighing out the samples and making the dilutions. During the actual assay—incorporating the samples into the diet and distributing the diet in cups—we use four workers for about 1½ h. Two workers can infest about five assays per hour, with one infesting and one capping the cups. Another two workers, working independently, read the previous week's assays at a rate of about five assays per hour. Cleaning glassware and laboratory tables and floors takes about 4 man-hours, while the necessary autoclaving and preparatory work for the next day's assay takes about 2 man-hours more. Total man-hours required for the assay vary between 25 and 30, depending on the experience of the workers. Since we obtain our eggs from a mass-rearing facility, we do not have to allow any time for rearing. However, our workers' time is not fully devoted to these bioassays, and we believe that if our three unskilled employees worked full time

and were used exclusively for the assay program, we would have no difficulty in maintaining a colony of the test insect suitable to our needs.

## CONCLUSION

The procedures we have described have worked well in our hands and have given us reproducible measurements of the potency of the *B. thuringiensis*  $\delta$ -endotoxins produced by several variants of this bacillus. We have used these same techniques in assays of the same endotoxins against other susceptible insects and in assays of the nuclear polyhedrosis virus of *Heliothis* species against the tobacco budworm. Data from these last assays are not yet extensive, but indicate that the procedures are working very satisfactorily. We believe that the techniques reported in this paper can be applied to a wide variety of assays with satisfactory results.

## LITERATURE CITED

- (1) Bonnefoi, A., Burgerjon, A., and Grison, P. 1958. Titrage biologique des préparations de spores de *Bacillus thuringiensis*. C. R. Acad. Sci. 247: 1418-1420.
- (2) Burgerjon, A. 1959. Titrage et définition d'une unité biologique pour les préparations de *Bacillus thuringiensis* Berliner. Entomophaga 4: 201-206.
- (3) Burges, H. D. 1967. The standardization of products based on *Bacillus thuringiensis*. In van der Laan, P. A. (ed.), Insect Pathology and Microbial Control, pp. 306-338. Proceedings of the International Colloquium on Insect Pathology, Microbial Control, Wageningen, the Netherlands, 1966. North-Holland Publishing Co., Amsterdam.

- (4) ———, 1967. Standardization of *Bacillus thuringiensis* products: Homology of the standard. *Nature* (London) 215: 864-865.
- (5) Daum, R. J. 1970. A revision of two computer programs for probit analysis. *Bull. Entomol. Soc. Am.* 16: 10-15.
- (6) Dulmage, H. T. 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti*. *J. Invertebr. Pathol.* 15: 232-239.
- (7) ———, 1973. Assay and standardization of microbial insecticides. *Ann. N.Y. Acad. Sci.* 217: 187-199.
- (8) ———, 1973. *B. thuringiensis* U.S. assay standard. Report on the adoption of a primary U.S. reference standard for assay of formulations containing the  $\delta$ -endotoxin of *Bacillus thuringiensis*. *Bull. Entomol. Soc. Am.* 19: 200-202.
- (9) ———, Boening, O. P., Rehnberg, C. S., and Hansen, G. D. 1971. A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit. *J. Invertebr. Pathol.* 18: 240-245.
- (10) McGarr, R. L., Dulmage, H. T., and Wolfenbarger, D. A. 1970. The  $\delta$ -endotoxin of *Bacillus thuringiensis*, HD-1, and chemical insecticides for control of the tobacco budworm and the bollworm. *J. Econ. Entomol.* 63: 1357-1358.
- (11) ———, 1972. Field tests with HD-1,  $\delta$ -endotoxin of *Bacillus thuringiensis* and with chemical insecticides for control of the tobacco budworm and the bollworm in 1970. *J. Econ. Entomol.* 65: 897-899.
- (12) Meehalas, B. J., and Anderson, N. B. 1964. Bioassay of *Bacillus thuringiensis*-based microbial insecticides. II. Standardization. *J. Insect Pathol.* 6: 218-224.
- (13) ———, and Dunn, P. H. 1964. Bioassay of *Bacillus thuringiensis*-based microbial insecticides. I. Bioassay procedures. *J. Insect Pathol.* 6: 214-217.
- (14) Raulston, J. R., and Lingren, P. D. 1972. Methods for large-scale rearing of the tobacco budworm. U.S. Dep. Agric. Prod. Res. Rep. No. 145, 10 pp.
- (15) Spittstoesser, C. M., and McEwen, F. L. 1961. A bioassay technique for determining the insecticidal activity of preparations containing *Bacillus thuringiensis*. *J. Insect Pathol.* 3: 391-398.
- (16) Van der Geest, L. P. S., and Wassink, H. J. M. 1972. Standardization of *Bacillus thuringiensis* preparation: A new bioassay method with *Pieris brassicae* as test insect. *J. Invertebr. Pathol.* 19: 361-365.

**END**