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POTENCY OF PRODUCTS BASED ON *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS*: INTERLABORATORY VARIATIONS

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ABSTRACT. Six quality-control laboratories in 4 countries independently bioassayed aliquots of a flowable formulation of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) against the international standard powder IPS-82. All laboratories substantially followed World Health Organization or U.S. Department of Agriculture standard protocols. Significant differences were found in resulting potency values between laboratories. Factors that may have influenced results, such as age, stage, and strain of larvae used, amount and type of food provided to larvae, and processing of samples were examined. Use of different rearing temperatures, different strains of *Aedes aegypti* L., or late 3rd instars vs. the recommended early 4th instars did not explain the inconsistencies. The slope of the dose–response curve of the IPS-82 powder was influenced by particle size, which varied with the nature and duration of sample homogenization. Laboratories using low-intensity processing obtained a greater slope in the dose–response curve for the flowable product than for the powder standard. The type and quantity of food provided to larvae affected susceptibility. Larvae fed an excess of protein-rich food became 4th instars in 3 days and were less susceptible to *B.t.i.* than those fed smaller quantities of carbohydrate-rich food that became 4th instars in 5–7 days. Overall, deviations from standard protocols with regard to larval stage, holding temperature, and lighting regime may not be as important as differences in sample processing and pretest rearing conditions. The need to improve standardization in these areas, which are not clearly specified in current protocols, is discussed.

KEY WORDS Bioassay, potency, standardization, *Bacillus thuringiensis* var. *israelensis*, mosquito, larvae

INTRODUCTION

Bacillus thuringiensis var. *israelensis* (*B.t.i.*) is a spore-forming bacterium that produces a proteinaceous incrustation in a crystalliferous body during sporulation. The crystal consists of at least 4 protein protoxins. Upon digestion by an insect larva having a sufficiently high midgut pH, the dissolved protoxins are enzymatically converted to the toxins that work more or less in concert (Federici and Wu 1994). If the larva is a mosquito or other susceptible dipteran species, the toxins attach to receptors in the midgut membrane and initiate a process ending in cell lysis. An individual insect dies when a sufficient amount of toxin is ingested and activated (Federici et al. 1990). Ultimately, the mortality in a population of susceptible larvae is dependent upon the quality and quantity of toxin ingested.

This characteristic of dose-dependence is used for determining the potency of a product based on *B.t.i.* A standard method has been developed in which a product of unknown potency is tested in parallel with a reference standard of known potency. A series of dilutions are made of both products, early 4th-instar mosquito larvae are exposed to the dilutions, and mortality is read after 24 h (de Barjac and Larget-Thiery 1984, McLaughlin et al. 1984, de Barjac 1985). The potency of the unknown can be calculated from the potency of the reference standard using a mortality difference ratio. In practice, we use the ratio of median lethal concentration (LC_{50}) values determined from log-probit-transformed dose–mortality data. The LC_{50} values are most accurately determined when a series of dilutions result in a distribution of mortalities both above and below 50%, but not reaching the extremes of 0 or 100%.

Theoretically, calculation of the potency of the unknown is not valid unless the unknown and the reference standard form parallel dose–mortality curves. However, the standard bioassay protocol does not stipulate a test of parallelism, and dose–mortality curves are often not parallel. Nevertheless, potency values are always provided on the labels of commercial *B.t.i.* products.

Certain other test parameters can significantly affect potency determinations of *B.t.i.* products. For instance, Skovmand et al. (1997) showed that the slope of the dose–response curve is inversely proportional to the size of particles in a formulation. When the aggregates of spores were nondestructively made smaller, slope increased, and vice versa. Among the commercially available products, most flowables have a small median particle size compared to powders. The international standard IPS-82 from Institut Pasteur (Paris, France) is a powder having a bimodal particle size distribution with peaks around 1.5 and 15 μm. In this study, we compare IPS-82 with a flowable formulation having a unimodal particle size distribution with a peak

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around 1.5 μm (Skovmand et al. 1997). Also, by having several laboratories bioassay aliquots of the same 2 samples, we can determine the extent of interlaboratory variation. The results will shed light on the value that the end user can attach to label potency values and may lead to more refined protocols to reduce variability.

MATERIALS AND METHODS

The following laboratories participated in this study: the quality control laboratory of Novo-Nordisk Plant Protection Division, Bagsvaerd, Denmark; the Institut Pasteur, Unité des Bactéries Entomopathogénes, Paris, France; EID-Mediterannée, Montpellier, France; ORSTOM–Bondy, Paris, France; KABS, Waldsee, Germany, in cooperation with the National Institute of Public Health, Praha, Czech Republic; and Benzon Research, Carlisle, PA, USA. These laboratories routinely perform large numbers of *B.t.i.* potency bioassays.

Mosquito larvae: Aedes aegypti L. larvae were used by all laboratories. The laboratories at EID, ORSTOM, KABS, and Institut Pasteur used the strain *bora bora* as recommended in the World Health Organization (WHO) protocol written by the latter laboratory. The laboratory at Novo-Nordisk used a strain originating from The London School of Hygiene and Tropical Medicine (London, United Kingdom). Benzon Research used a strain that originated from Gainesville, FL. All laboratories used early 4th instars, except EID, which used either late 3rd instars or early 4th instars that were 24 h older. Fifty to 100 larvae were used per concentration for each replicate (Table 1).

Rearing: The standard protocols provide no guidelines for rearing conditions prior to bioassay, and many types of food were used, such as desiccated liver powder, aquarium fish flakes, and dog and cat food pellets, either powdered or introduced whole into the water for gradual dissolution.

At Novo-Nordisk, additional tests were performed to determine the influence of the food provided during rearing on LC_{50} values. A mixture of pulverized dog biscuit and 20% fish food or whole cat pills (a type of compressed, protein-rich food pellet) were compared.

All larvae were reared and tested at 25–27°C, 12:12 h light : dark (LD), except at Institut Pasteur and Benzon Research where 14:10 h LD was used.

Bioassay: A quantity of the international standard IPS-82 was delivered to each participating laboratory by Institut Pasteur. This standard is a lyophilized powder containing 1.8–2.8 × 10^{11} spores/g. The flowable product was provided by Novo-Nordisk and contained ca. 1 × 10^{10} spores/g. The product was formulated with a high level of glycol and polyglycol to obtain a product that would not freeze at −18°C. This product was intended to serve as an internal standard at Novo-Nordisk. The product was sealed in ampoules each containing 1.5

Table 1. Preparation of bioassay test suspensions of the standard IPS-82 and a flowable formulation, and administration to mosquito larvae by 6 laboratories.

Laboratory	Sample suspension	Homogenization	Agitation of final dilutions	Quantity transferred to test cups (ml)	Test cup volume	Dilution ratio	No. of cups/concentration	No. of larvae/cup
Benzon Research	IPS-82: 50 mg in 100 ml Flowable: 1 g in 200 ml	Glass bead mill, 5 min	Magnetic stirrer	0.50–1.24	100	0.80	3	20
EID	IPS-82: 20 mg in 50 ml Flowable: 300 mg in 50 ml	Rod homogenizer, 10 min	Vortexer	0.167–1.00	250	0.67–0.75	4	25
KABS	IPS-82: 10 mg in 200 ml Flowable: 50 mg in 250 ml	Glass bead mill, 10 min	Magnetic stirrer	0.15–0.30	100	0.50	4	25
Novo-Nordisk	IPS-82: 20 mg in 50 g Flowable: 1 g in 50 g	Rod homogenizer, 30 sec	Shaking	10.00	100	0.80	3	20
ORSTOM	IPS-82: 100 mg in 1,000 ml Flowable: 1 g in 1,000 ml	Glass bead mill, 30 min	Shaking	3.12–50.00	100	0.50	4	25
Institut Pasteur	IPS-82: 100 mg in 10 ml Flowable: 100 mg in 10 ml	Glass bead mill, 10 min	Vortexer	0.015–0.120	150	0.50–0.75	2	25

Table 2. Median lethal concentrations (LC_{50}s) of IPS-82 and a flowable *Bacillus thuringiensis* var. *israelensis* formulation, and the calculated potency of the flowable product.[1]

	Benzon Research	EID	KABS	Novo-Nordisk	ORSTOM	Institut Pasteur
n	20	17	12	25	5	15
LC_{50} of IPS-82 (mg/liter ± SD)	0.0217 ± 0.0048c	0.0106 ± 0.0016b	0.0125 ± 0.0036b	0.0063 ± 0.0012a	0.0122 ± 0.0021b	0.0117 ± 0.0056b
LC_{50} of flowable product (mg/liter ± SD)	0.405 ± 0.082c	0.280 ± 0.037b	0.222 ± 0.071b	0.144 ± 0.032a	0.261 ± 0.020b	0.251 ± 0.125b
Potency of flowable product (ITU/mg ± SD)	808 ± 119bc	570 ± 55a	854 ± 117c	628 ± 76a	697 ± 96ab	628 ± 234ab

[1] Values in the same row followed by the same letter are not significantly different (analysis of variance, $\alpha = 0.05$).
[2] Based on an assigned IPS-82 potency of 15,000 ITU/mg.

g, and it was stipulated that at least one ampoule should be used for each bioassay replicate. The laboratories were asked to perform the bioassays according to their usual routine and to describe this routine in detail.

Institut Pasteur tested for interampoule variation in the flowable sample by simultaneously testing 5 mixtures of 2 ampoules each, with 5 replicates using the same mixtures performed on 5 different days. Novo-Nordisk performed 5 sets of bioassays in 1 day to evaluate the intraday variation, and 26 other bioassays on different days using pairs of standard and flowable samples. The other laboratories carried out 1 or 2 tests per day with a new ampoule of flowable sample each day.

Some variation occurred in how samples were processed. At Novo-Nordisk, 50 mg of the standard IPS-82 were allowed to stand in water with a droplet of wetting agent (salt of polymer of carboxylate) for 30 min, then homogenized and further diluted to produce a series of test dilutions. Benzon Research also added a wetting agent (0.2% Tween 80) to the suspension before homogenizing. No other laboratories used wetting agents. At all laboratories, the flowable product was mixed with water to provide a stock from which other concentrations were prepared. No other homogenization was necessary. A summary of dilution and homogenization procedures is given in Table 1.

None of the laboratories provided food during the bioassays. Mortality was observed 24 h posttreatment.

Raw data and protocols from each laboratory were provided to the first author for analysis. Bioassay mortality data were log-probit transformed, analyzed by linear regression, and the slope of the transformed dose–mortality data was calculated. A chi-square test was used for the validation of iterative approaches to obtain the best fit of the regression line (Finney 1971). A one-sided analysis of variance (ANOVA) was carried out on each bioassay to evaluate goodness-of-fit to the probit model. Nonvalid tests were discarded ($P > 0.10$).

Finally potency of the flowable product was calculated for each bioassay using the formula

$$\text{potency(Fl)} = \frac{LC_{50}(\text{Std})}{LC_{50}(\text{Fl})} \times \text{potency(Std)},$$

where Std is the international standard IPS-82 assigned a potency of 15,000 ITU/mg (de Barjac and Larget-Thiery 1984) and Fl is the flowable test product.

RESULTS

The calculated potency values of the flowable *B.t.i.* product based on data provided by the 6 participating laboratories are given in Table 2 and Fig. 1. The values range between 570 and 854 ITU/mg. Some differences between laboratories are significant, as noted ($P < 0.01$, df 5,88). The relatively

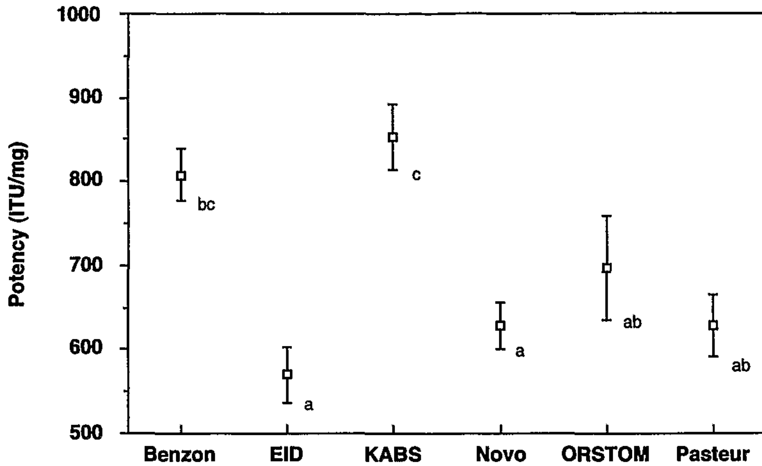

Fig. 1. Potency of a flowable *Bacillus thuringiensis* var. *israelensis* product as determined by 6 laboratories. Error bars indicate standard deviations. Points labeled with the same letter are not significantly different ($\alpha = 0.05$).

large confidence limit of the ORSTOM mean value was due to a low number of valid determinations (5 compared to 12–27 from the other laboratories).

Potency is based on the ratio between the LC_{50} of the standard and the LC_{50} of the flowable sample. Therefore, the potency variations may be caused by a variation in one or both parameters. Analysis of variance showed the LC_{50}s of both products varied significantly between laboratories (Table 2, $P << 0.001$, df 5,97 for the test product; $P << 0.001$, df 5,74 for the standard). The LC_{50} values from EID, KABS, ORSTOM, and Institut Pasteur were not significantly different from each other. Benzon Research obtained higher LC_{50} values and Novo-Nordisk lower LC_{50} values than the other 4 laboratories.

The slope of the dose–mortality curve of the flowable product was significantly different than that of the standard at Benzon Research, KABS, and Novo-Nordisk (Table 3, $P < 0.001$) but was not significantly different at the other 3 laboratories. The lack of difference at ORSTOM and Institut Pasteur may have been due to relatively low replication. Where the slopes were different, the slope of the flowable sample was higher than that of the powder standard.

The EID laboratory compared the use of late 3rd instars with early 4th instars. The LC_{50} values of the standard and the flowable sample were slightly (10%) but not significantly higher using 4th instars (for the standard, $P > 0.10$, $t = 0.89$, df 15; for the flowable sample, $P > 0.25$, $t = 0.16$, df 15). Accordingly, no differences were found in the potency estimates ($P > 0.25$, $t = 0.0012$, df 15) or in the slopes of the dose–mortality curves (ANOVA, $P << 0.001$, $F = 0.3$, df 24).

When Institut Pasteur tested for interampoule variation in the flowable sample, 1 of the 5 replicates was lost due to lack-of-fit in the standard. Analysis of variance shows that no interaction occurred between sample and test day ($P > 0.05$, $F = 0.29$, df 4,12). Novo-Nordisk and Institut Pasteur found greater variation in the LC_{50} of the flowable sample using results from all bioassays (the coefficient of variation, defined as the quotient of the standard deviation/mean = 22% for both labs) than from multiple bioassays performed on the same day (coefficient of variation = 8% for both labs). The coefficients of variation for the LC_{50} of the standard on different days were 33 and 40%, respectively.

At the Novo-Nordisk laboratory, the type of food provided to larvae during pretest rearing affected both the LC_{50} and the slope of IPS-82. A mixture of dog biscuit and fish flakes resulted in an LC_{50} of 0.0071 μg/ml water vs. 0.0094 μg/ml water (Table 4, $P < 0.01$, t-test) for cat pills. The slopes of the dose-mortality curves were 3.81 and 2.27, respectively (Table 4, $P < 0.001$, t-test). Data on the flowable product were not obtained in this test.

The time required for larvae to reach 4th instars varied according to the provided diet. At Benzon Research, where dessicated liver powder was used, only 3 days were required. At Novo-Nordisk, using less protein-rich dog biscuits, 7 days were required. At most other laboratories, using cat pills, 5–7 days were required.

DISCUSSION

When potency bioassays were performed at 6 different laboratories on identical samples of a flowable *B.t.i.* product, the results were not consistent. Significant differences were found in the estimated potencies (Fig. 1 and Table 2) and in LC_{50}s of both the flowable product and the international standard IPS-82 (Table 2). Between laboratories, the variation in the LC_{50} was greater for the standard than for the flowable product (Table 2). Within laboratories, the variation in the LC_{50} of the stan-

Table 3. Comparison of slopes of dose mortality curves for the standard IPS-82 and a flowable formulation.

	Benzon Research	EID	KABS	Novo-Nordisk	ORSTOM	Institut Pasteur
IPS						
Slope ± SD	4.42 ± 0.56	5.26 ± 0.59	3.65 ± 0.47	3.86 ± 0.28	3.12 ± 0.26	5.75 ± 0.46
n	20	17	12	25	5	4
Flowable product						
Slope ± SD	5.27 ± 1.10	5.39 ± 1.12	4.19 ± 0.65	4.55 ± 0.60	3.81 ± 0.24	5.17 ± 1.09
n	20	17	12	27	5	21
Slopes equal? ($\alpha = 0.05$)	No, $P < 0.001$	Yes, $P \gg 0.10$	No, $P < 0.001$	No, $P < 0.001$	Yes, $P = 0.10$	Yes, $P = 0.10$

dard was larger than that of the flowable product. Accordingly, most variations found in potency determinations were due to variation in the LC_{50} of the standard. Most laboratories with a high number of bioassay replicates showed a significantly steeper slope in the dose-response curve for the flowable product than for the standard.

Many explanations are possible for these interlaboratory differences. Although a thorough examination of the factors involved was beyond the scope of this study, certain differences in methods and materials were influential. For instance, given the demonstrated influence of particle size on slope (Skovmand et al. 1997), we would expect that the method of homogenization of the IPS-82 powder would influence slope. The powder consists of aggregates of *B.t.i.* spores and crystals, and intense or prolonged homogenization decreases the median particle size. Homogenization would not influence slope of the flowable product, as it was formulated at the single cell/crystal level and could not be divided further. When stored in a freezer, the flowable product would not increase in particle size because it was formulated with a high level of antifreeze agent and tested for nonfreezing characteristics.

Table 1 gives details on sample preparation and handling for each laboratory. Two laboratories used high-speed rotor/stator rod homogenizers but with different treatment times. Novo-Nordisk, using a short treatment time of 30 sec, reported a significant difference in slope between the flowable product and the standard. The EID laboratory, using a long treatment time of 10 min, did not find a significant difference. Novo-Nordisk analyzed actual particle size distributions, and found that the volume median diameter of particles was 1.5 μm for the flowable product, and that the standard exhibited a bimodal distribution with one peak at 1.5 μm and another larger peak at 15 μm (Skovmand et al. 1997). We believe that duration of homogenization at EID reduced the larger aggregates of particles, resulting in a size distribution more like that of the flowable product. The other laboratories homogenized samples by introducing glass beads into a suspension and rotating on a shaker table or similar device for between 5 and 30 min. This method provides relatively little shearing action and serves mainly to break up large aggregates. Two of the laboratories using this method, Benzon Research and KABS, obtained significant differences in slope between mortality curves of the powder standard and flowable sample.

Substantial variation may occur in the degree to which particle size reduction occurs even when efforts are made to standardize the method of homogenization. If this is true, we would expect that the LC_{50}s and slopes of IPS-82 powder would exhibit more within-laboratory variation than for the flowable product. This was indeed the case at all laboratories.

A significant correlation was found between the

Table 4. Influence of larval rearing food on the median lethal concentration (LC_{50}) and slope of dose–mortality curve for the standard IPS-82.

	Dog biscuit + fish flakes (n = 12)	Cat pills (n = 5)	Level of significance[1]
$LC_{50} \pm SD$	0.0071 ± 0.0026	0.0094 ± 0.0021	$P < 0.01$
Slope ± SD	3.81 ± 0.70	2.27 ± 1.01	$P < 0.001$

[1] Student t-test for comparison between food regimes.

slope of the standard and that of the flowable sample across laboratories (Table 3, $P < 0.05$, df 4, $r^2 = 0.88$). This indicates that slope is not only influenced by particle size and other factors related to the product, but by bioassay parameters such as homogeneity of test larvae. The test at EID did not show significant differences in susceptibility or slope between late 3rd and early 4th instars, so a few 3rd instars in the test cups that are supposed to contain only 4th instars should not materially affect the results of bioassay. However, Benzon Research reported that using 30–50% 3rd instars in that laboratory reduces the LC_{50} by 20–30% compared to using 100% early 4th instars, but that potency determinations were not significantly affected (unpublished data).

The different strains used for these bioassays could not explain variations in slope, because ORSTOM and KABS, with the lowest slope values, and EID and Institut Pasteur, with much higher values, used the same strain originating from Institut Pasteur, *Ae. aegypti* strain *bora bora.* However, the LC_{50} may have been influenced by the mosquito strain used. The 4 laboratories using strain *bora bora* obtained similar LC_{50}s (Table 2). The 2 laboratories using other strains found significantly different LC_{50}s. De Barjac and Coz (1979) showed that the *bora bora* strain was less susceptible to *B.t.i.* than were various other strains tested at the same laboratory. We have not determined whether our results are due to use of the same strain or because the 4 laboratories using the *bora bora* strain all reared their larvae to test size within 5–6 days using very similar rearing methods.

A correlation was found between the number of days required to reach 4th instars and the LC_{50} across laboratories (Fig. 2). This correlation may be caused by rearing densities and feeding conditions. Benzon Research provided protein-rich liver powder in quantities such that food was never depleted. This resulted in a development time of just 3 days. Novo-Nordisk used a carbohydrate-rich dog biscuit mixture in relatively low quantities, resulting in a development time of 7 days. A more protein-rich diet (cat pills or liver powder) possibly results in mosquito larvae that search less for bacteria during the test period, or are less susceptible to the toxins than those fed smaller quantities of less protein-rich diet. Dog biscuits contain much fibrous matter resulting in larvae defecating mostly undigested food wrapped in the peritrophic membrane. Tests at Novo-Nordisk compared results of bioassays using larvae that were reared on 2 different diets (Table 4). The LC_{50} was higher and the slope was lower when the test larvae were fed cat

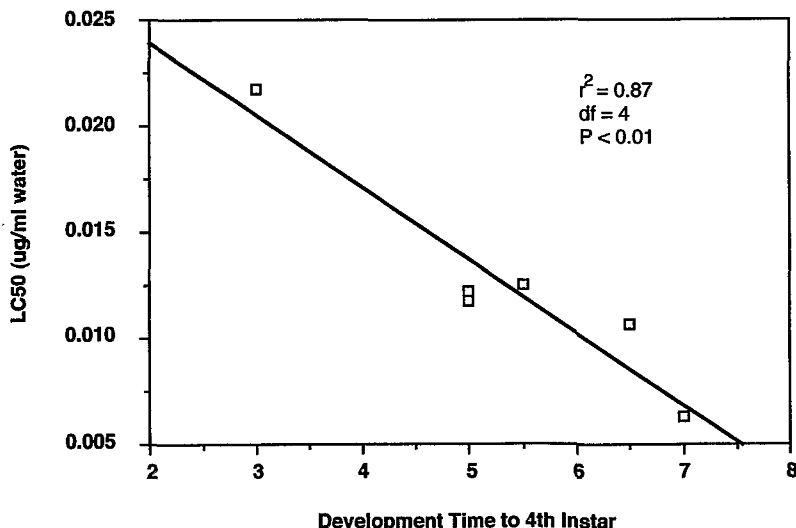

Fig. 2. Median lethal concentration (LC_{50}) of the standard IPS-82 vs. larval age at the start of bioassays.

pills compared to larvae fed with a mixture of dog biscuit and fish flakes.

Novo-Nordisk and Institut Pasteur examined the variation in LC_{50} for multiple bioassays performed on the same samples on the same day vs. different days. Both laboratories obtained a coefficient of variation of 8% for same-day bioassays and 22% for different-day bioassays. These results support the standard protocol recommendation that bioassay replicates should be carried out on different days.

Much of the bioassay data obtained by ORSTOM and some from KABS were discarded due to insufficient numbers of observations between 0 and 100% mortality. These laboratories used a dilution factor of 0.5 (i.e., each concentration used was 0.5 times the next higher concentration) that often resulted in more than one observation of 0% and/or 100% mortality. The standard protocol recommends a dilution factor between 0.75 and 0.80, which is more suitable for the slope of dose–mortality curves found with most *B.t.i.* products.

Overall, deviations from the WHO and U.S. Department of Agriculture standard protocols with regard to larval stage, holding temperature, and lighting regime, may not be as important as differences in pretest rearing conditions. Variation in rearing protocols were thought to affect the reference standard and the tested products equally (de Barjac 1985), but our results refute this.

We conclude that a standard test protocol should specify larval rearing conditions such as type of food and feeding schedule, larval density (such as that suggested by de Barjac and Larget-Thiery 1984), sample treatment methods, the size of bioassay cups, and the density of larvae in each cup. The latter parameter was shown to be important by several authors (Mulla et al. 1990, Becker et al. 1992). For sample treatment, several possibilities exist. Samples may all be homogenized to the single-cell level to reduce variation between laboratories and product types, but the influence of this treatment will be greater for some products (such as powders) than for others. As an alternative, 2 standards may be used: a flowable standard with flowable products and a powder standard with powder products or products produced with powders, for example, most granules. This, however, may contribute to a general confusion about the meaning and value of bioassays. The 2 standards would have to be intercalibrated by a nonbioassay method.

Biochemical or immunologic methods have been developed to assay crystal protein concentration (Skovmand and Sterndorf 1994). Thus far, such tests have failed to provide a reliable correlate to killing power and are incapable of determining the bioavailability of toxin (e.g., particle size for mosquito larvae) or to distinguish between active and inactive protein. The latter problem may be solved with development of more specific methods, but the problem of bioavailability is unlikely to be solved by biochemical means. On the other hand, chemical pesticides are labeled with the concentrations of active ingredients, even though differences in formulation can have a major impact on efficacy given the same concentration of active ingredient. This problem is mostly solved by specifying an appropriate field application rate. Such information is already required on a pesticide label whether the product is chemical or microbial.

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