# Parameters Influencing Potency of *Bacillus thuringiensis* var. *israelensis* Products

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**ABSTRACT** Bioassays of products based on *Bacillus thuringiensis* var. *israelensis* have been carried out according to standard protocols. These analyses revealed that the slopes of the log-probit transformed concentration mortality curves of various products were different from that of the international standards (IPS82 for *B. thuringiensis israelensis*). For statistical reasons, this invalidates the tests. Products giving various slopes of the concentration mortality curves will obtain different potencies when estimated at a $LC_{90}$ level than when estimated at $LC_{50}$ level, as normally done. The $LC_{90}$ level is probably more relevant for the field effect. Changing the median particle size of a product in a non destructive way results in change of slope and $LC_{50}$ and thereby potency. Therefore, potency of a product as measured in these bioassays is not just a measure of the quantity of *B. thuringiensis israelensis* crystal protein present, but a function of product parameters like median particle size. Biochemical methods for quantification of toxin can therefore not relate simply to potency of the products obtained with this method. It is suggested that standard protocols for bioassay may be changed to assure equal particle size of products and samples to obtain parallel dose response.

**KEY WORDS** *Bacillus thuringiensis* variety *israelensis*, bioassay, dosemortality, particle size



*Bacillus thuringiensis* VAR. *israelensis* is an entomopathogenic bacterium that produces a proteinaceous incrustration in a crystalliferous body (i.e., the protein crystal or delta-endotoxin). The protein crystal of *B. thuringiensis israelensis* consists of a protoxin that is dissolved and then enzymatically activated in the midgut of the mosquito larvae (Charles and de Barjac 1981). Because of its high degree of specificity and no known vertebrate toxicity of commercial strains, *B. thuringiensis* has been used commercially for many years (but see Damgaard 1995). In recent years, a better understanding of the mode of action and genetic techniques has expanded, and the use and our basic knowledge of the bacterium and especially of the delta-endotoxins has improved (reviewed by Höfte and Whiteley 1989, Knowles et al. 1989).

Products based on the most commonly used strains of *B. thuringiensis* are given a potency based on bioassays on insts. Concentration–mortality data are obtained, transformed to a log-probit scale, and potency is obtained by comparing the estimated $LC_{50}$ of a test substance with that of a standard with a known potency (Dulmage 1973, de Barjac 1985). The rationale is that daily within-laboratory or interlaboratory variations can be accounted for by using relative estimates. The standards are internationally recognized standards (e.g., HD-1-S-1980 for *B. thuringiensis kurstaki* and HD-968-S-1983 and IPS82 for *B. thuringiensis israelensis*).

To reduce the interlaboratory variation, protocols were also suggested to standardize the bioassays (McLaughlin et al 1984, deBarjac 1985). These protocols were used when a number of laboratories set the potency of the current *B. thuringiensis israelensis* standards. Nevertheless, the value setting of the *B. thuringiensis israelensis* standards did reveal interlaboratory variation. The reasons for these variances were not analyzed (McLaughlin et al. 1984).

It is a statistical prerequisite for the comparisons of $LC_{50}$ values that the concentration–mortality curves are parallel (e.g., that the slope of the concencentration mortality curves [slope for short below] are the same). This requirement is of course the same whether commercial *B. thuringiensis* products, single toxins, dissolved toxins, or effect of extracts from genetically engineered plants are compared. When comparing a standard and a test sample, the difference in slope of the 2 curves has to be large to be statistically different because of the rather great variance of bioassays. Accordingly, the criteria are often met and many authors do not analyze to ensure that such criteria are met.

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Tabashnik et al. (1993) analyzed slope and $LC_{50}$ data from 54 bioassays on 15 populations of *Plutella xylostella* (L.) and concluded that the variation in slope in bioaasays is not biologically meaningfull. The problem with this and other studies on bioassay methods is that the amount of data used was too small compared to the variation of the bioassay to see if slope was a product-dependent parameter.

This study contains bioassay data of commercial products and test formulations with *B. thuringiensis israelensis* collected over 4 yr, ≈1,500 bioassays. Average slope of various product types are analyzed to see if the average values are different from that of the standard, IPS82, and if they are product or product type dependent. The slope of the products are related to particle size of the products and the particle size is changed experimentally to show the effect on the slope of the dose mortality curves. The consequences of that for the measured potency are demonstrated.

The results are discussed in relation to the thorough investigations carried out by the Working Group on Biological Control of Vectors in WHO, which tried to standardize test methods and determine potency of various proposed international standards (WHO reports and personal communications 1979–1981 from the Working Group in which ORSTOM participated).

## Materials and Methods

**Mosquito Assays.** The *A. aegypti* strain obtained from K. Arevad (The Danish Pest Infestation Laboratory) in 1987 were used for the bioassays in Copenhagen. The strain originated from The London School of Hygiene and Tropical Diseases.

Larvae were fed with a mixture of ground dog biscuits and fish flakes used to feed fish in aquaria. Larvae were reared at 27°C and a photoperiod of 12:12 (L:D) h, and early 4th instars were used for the testing, except when specified. Results of the bioassays were read after 24 h. The protocol was identical to McLaughlin et al. 1984, although waxed cups were replaced by 150-ml plastic cups. Bottled water was used for rearing and testing because it was free of chlorine and its quality was constant.

We tested wettable powders of Bactimos WP (Novo Nordisk, Bagsvaerd, Denmark), and primary powders Vectobac TP (Abbot, Chicago, IL), Primary Powders (Novo Nordisk), and the international standard IPS82 (Institut Pasteur, Paris). The flowable products tested were Acrobe (Becker Microbials Products, FL), Skeetal FC (Novo Nordisk), Teknar HPD (Sandoz, Zürich), Vectobac 12AS and Vectobac FC (Abbot) and a number of technical products from Novo Nordisk: NN-1, Sludge-1, Sludge-2. The sludges were unformulated, concentrated beers, and NN-1 was an internal, fluid standard also used for biochemical potency control.

Powder samples were allowed to stand in water for 30 min and a droplet of a wetting agent was added if the sample was not allready formulated. The sample was then homogenized (IKAmag EOA5 Homogenizer with Polytron PTA 205 shaft) for 30 s and further diluted to produce a series of test dilutions. Flowable products were homogenized in water (Warren blender) for 30 s and then diluted further to produce the serial dilutions. These homogenization steps did not disintegrate particle size of the tested products. From these dilutions, 10-g samples were transferred into 150-ml cups with 90-g of water and 20 mosquito larvae.

To examine the feeding activity of the larvae surviving test concentrations, they were carefully sampled with a pipette and transferred to another cup with water. Finely ground charcoal powder was added and the larvae were picked up after 30 min and squeezed between 2 glass plates for the microscopical examination of the gut content.

Concentration-mortality data were log-probit transformed, analyzed by linear regression and the slope of the (transformed) dose mortality data was calculated. A 1-sided analysis of variance verified that the data actually fit a straight line. Nonvalid tests were discarded.

For potency calculations, we used the internationally recognized standard for mosquito assay, IPS82 (15,000 ITU/mg) provided by Institute Entomopathogene, Institut Pasteur, Paris, France. Standard vials are kept at −18°C in our laboratory.

Product potency was calculated by:

$$Potency(A) = Potency(STD) \cdot \frac{LC_{50^{(STD)}}}{LC_{50^{(A)}}},$$

where (A) is the product and (STD) is the standard.

Each sample was bioassayed at least 3 times on various days and the results presented are average values: log transformed for $LC_{50}$ and potency values, arithmetric means for slope.

**Physical Analysis.** Particle size was measured by laser diffractometry on a Sympatec Helos particle size analyzer in Novo-Nordisk, Bagsvaerd, or in Entotech, Novo-Nordisk, Davis, California. One of 2 lenses were used for final particle size analysis: one covering from 1 to 1,000 $\mu$m or one covering from 0.1 to 100 $\mu$m. In this way we obtained a best match between lens and particle size distribution. For each product, at least 2 samples were weighed and suspended in water, and for each, sample at least a double determination of particle size was made. The printouts of the 2 machines were slightly different, one giving a grid scale, the other not. Both machines made automatic scale corrections. For the y-axis this implied that curves with a high number in the scale are more narrow that those with a low number of the scale.

**Table 1. Slope of the log-probit transformed dose mortality data from 3 yr**

| Product/yr | 1990 slope[a] | *n* | 1991 slope | *n* | 1992 slope | *n* | 1993 slope |
|---|---|---|---|---|---|---|---|
| IPS 82[b] | — | 0 | 3.85 ± 1.01 | 33 | 3.87 ± 0.73 | 71 | 3.81 ± 0.51 |
| Flowables[c] | 4.12 ± 0.75 | 32 | 4.33 ± 0.77 | 50 | 4.17 ± 0.87 | 50 | 4.22 ± 0.55 |
| Powders | 3.20 ± 0.58 | 37 | — | 0 | 3.42 ± 0.98 | 141 | 3.42 ± 0.84 |

Analysis of all data (SAS Institute 1989) showed a significant difference between the mean values of the slopes ($P < 0.01$). There was no significant difference within the years for the product groups ($P > 0.10$), but none of the groups had the same slopes ($P < 0.01$).

[a] An internal powder standard was used in 1990 and represents all powder data from that year.

[b] IPS82 is the international standard for *B. thuringiensis israelensis*.

[c] The flowable data represents products and fermentations that have not been stored above 5°C.

Particle size was manipulated by sonicating powder products or freezing fluid samples. The powder was dispersed in water in a plastic beaker, agitated, and allowed to stand for 30 min. The beaker was then put in an ice bath and the sample sonicated at low intensity. In a preliminary test, a primary powder was sonicated in 0, 30, 60, 90, 120, 240, and 480 s. From this experiment of sonication times, 0, 120, and 480 s were selected, giving 3 different particle size ranges. Nine bioassays were carried out with 2nd and with 4th instars on the sonicated powders of the type used in the preliminary test. Three tests were carried out with another type of primary powder.

The fluid products were stored in a freezer at −18°C overnight to allow for formation of crystallic ice in the samples. Fast freezing in liquid nitrogen and freezing of a product with a high content of glykol did not result in a change of particle size and are not used for further analysis here. Samples analyzed for particle size distribution were also bioassayed and slope and potency determined. Frozen samples were rethawed and homogenized for 30 s before analysis.

## Results

The slope of the concentration–mortality curves in tests with IPS82 averaged 3.86, based on data from 1990–1993 (Table 1). Corresponding figures for flowable products based on concentrated sludge and for powder products (spray dried) were—with a few exceptions—in the range of the mean values of 4.21 and 3.31, respectively. These values were significant different ($P<0.01$).

Most of the fluid products had a narrow particle size distribution with a median particle size ≈10 μm. The exception was Acrobe with a large particle range (Table 2; and Fig. 1 [note y-scales, the peak of Acrobe is actually 3 times lower than the peak of Skeetal].). By contrast, the powders had a higher particle size and larger variation (Table 3; Fig. 1). The exception was Vectobac TP which had a small particle size similar to that of the flowables, but a larger particle size range.

In general, fluid samples produced higher slopes in bioassays than powder samples. However, 2 exceptions were informative: Acrobe had a low slope and Teknar TP had a high slope. For all samples, there was a negative correlation between slope and median particle size (Fig. 2).

Before sonication, the tested primary powders had a median particle size close to 40 μm. The preliminary test of a primary powder showed that it broke up in 2 fractions with smaller, 1–2 μm, and larger, 30–40 μm particles. Extending sonication time resulted in a higher frequency of small particles and a significant lower frequency of the large particles; at 480 s, almost no large particles remained (Fig. 3). Experiments were continued using sonication times of 0, 120, and 480 s, which gave almost the same pattern of particle size distribution each time except when applied to another type of primary powder. This powder proved to be much more susceptible to sonication. After 120-s sonication, median particle size was 3 μm

**Table 2. Median volume of particle size of commercial and laboratory products**

| Sample | Median vol, μm | Distribution of particles | Slope ± SE |
|---|---|---|---|
| Skeetal | 12 | 1 peak | 4.37 ± 0.93 |
| Teknar HPD | 9 and 50 | 2 peaks | 3.60 ± 0.97 |
| Vectobac 12AS | 11 | 1 peak | 3.64 ± 0.41 |
| Acrobe | 5 | broad range | 3.80 ± 0.96 |
| Unformulated | 7–8 | 1 peak | 4.40 ± 0.85 |
| Fluid Standard | 3.8 | 1 peak | 4.84 ± 0.51 |
| Primary Powder | 38–41 | 1 peak | 3.63 ± 1.0 |
| IPS 82 | 1.5 and 15 | 2 peaks | 3.87 ± 0.73 |
| IPS82, light sonicated | 1.5 and 15 | 2 peaks | 3.91 |
| IPS82, intense sonicated | 0.5–1.5 | 2 peaks | 5.1 |

When the product had particles distributed in two size classes, the median of each class is indicated. When various products of the same type were tested, the range of peak values are given.

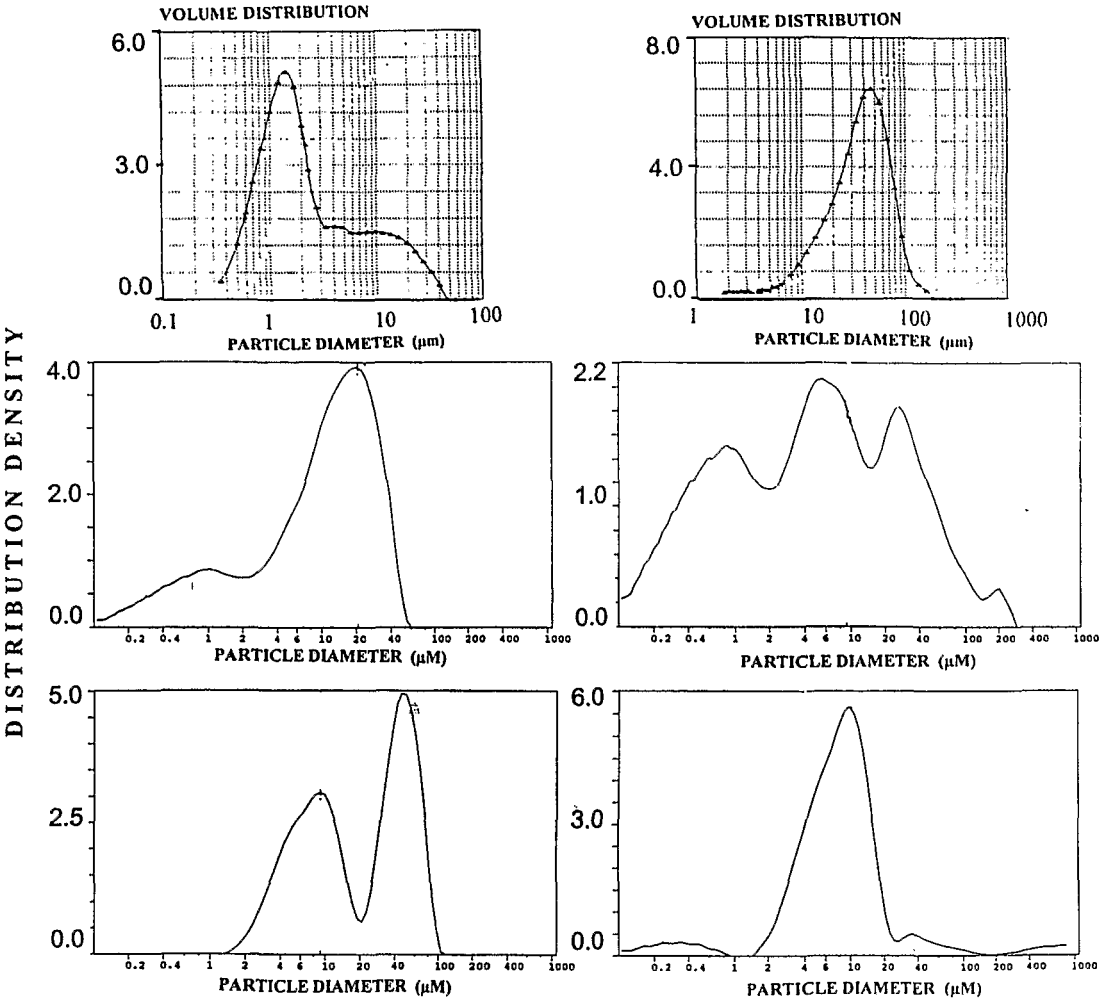


**Fig. 1.** Particle size distribution of some of the tested *B. thuringiensis israelensis* powders after light (0.3 W) homogenization in water; (A) Vectobac TP [R], (B) Bactimos WP, (C) Vectobac FC, (D) Acrobe FC, (E) Teknar FC, and (F) a concentrated sludge.

and there were no big particles. After 480 s, median particle size was 2 $\mu$m.

For the 2nd and for the 4rth instars, potency decreased and slope increased with sonication time (Table 4; Fig. 3). Bioassays with the 2nd type of powder showed that $LC_{50}$ increased so much that a partial destruction of the *B. thuringiensis israelensis* crystal was suspected (Table 4).

Sonication was also applied to the standard, IPS82. Without sonication, IPS82 consisted of 2 fractions of particles; small particles with a mean size of 1.5 $\mu$m and larger particles of $\approx$15 $\mu$m. A light sonication (2 W for 30 s) reduced particle size only sligthly and had no influence on the bioassay. More intense sonication (20 W for 30 s) reduced the number of larger particles and increased the

**Table 3. Average $LC_{50}$, slope, and potency of products tested in bioassays**

| Product | $LC_{50}$ ± SE | Slope ± SE | *n* | Potency ± SE |
|---|---|---|---|---|
| Skeetal FC | 0.123 ± 0.038 | 4.12 ± 0.86 | 41 | 830 ± 200 |
| Teknar HPD | 0.0911 ± 0.0271 | 3.67 ± 0.81 | 15 | 1,200 ± 200 |
| Vectobac TP 1993 | 0.0108 | 4.78 | 3 | 8,200 ± 1,800 |
| Vectobac 12AS | 0.107 ± 0.018 | 3.64 ± 0.41 | 4 | 950 ± 180 |
| Acrobe | 0.327 ± 0.165 | 3.50 ± 0.70 | 5 | 390 ± 110 |
| Bactimos WP | 0.0256 ± 0.0055 | 3.64 ± 1.02 | 40 | 3,600 ± 950 |
| IPS 82 | 0.00672 ± 0.00160 | 3.82 ± 0.83 | 115 | 15,000 |

*n*, number of bioassays.

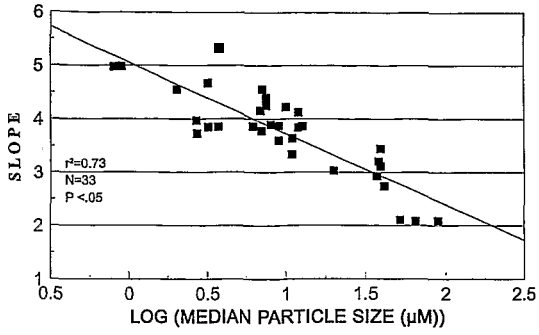


**Fig. 2.** Correlation between median particle size of a product and slope of the dose mortality curve when the product is bioassayed. All products and particle size manipulated products based on 3 or more bioassays are included in the table.

number of smaller particles (Fig. 4), but it did not change the slope though the $LC_{50}$ nearly doubled.

The sonication, particularly at high intensity, could have resulted in a partial destruction of the toxin (e.g., by splitting the crystal into subunits). Therefore, we evaluated a nondestructive method to change particle size. Slow freezing of a protein solution is known to result in flocculation causing larger particles.

Five formulated (Skeetal and Vectobac) and 5 unformulated samples were frozen (-18°C), $LC_{50}$ and slopes were measured before and after freezing. Freezing resulted in decreased slope $LC_{50}$s. The effect was smaller for the formulated products than for the unformulated (Table 5).

To confirm that this effect in the bioassay was caused by induced changes in the products, a sludge was split in 2 and 1 of these formulated. The formulated and the nonformulated were further split in 2 and 1 of each was frozen (-18°C), the other was not. In this way, we obtained a frozen, formulated sludge, a nonfrozen, formulated sludge, a nonformulated, frozen sludge and a nonformulated, nonfrozen sludge, all of the same origin. The 4 samples were tested in bioassay and for particle size, except for the formulated sample where the tests on the frozen sample mistakenly was not tested. Freezing increased particle size, and decreased slope and potency. The effect was most pronounced for the unformulated sludge (Table 6).

After a bioassay of the standard, surviving mosquito larvae were transferred to clean water and allowed to ingest charcoal powder particles for 30 min. At all concentrations, a few larvae did not ingest anything and died, but most of the larvae fed vigorously and filled the oesophagus with charcoal powder particles within 30 min. There was no correlation between the amount of charcoal powder particles taken in and the previous test concentration (data not shown). Larvae from medium test concentrations often had filled their gut with macerated pieces of their dead cup-fellows.

## Discussion

Calculations for a large number of bioassays in this study showed that the concentration–mortality curves were often not parallel, but the slopes of the curves were dependent on the product type. In general, fluid products have steeper slopes than powder products (Tables 2 and 3) with 2 informative exceptions: a flowable with a wide particle size distribution gave a low slope and a powder with a small median particle size gave a high slope. Therefore, the slopes are probably a function of particle size/distribution and not of product type.

In principle, products giving different slopes in dose effect studies cannot be compared (Finney 1971), which put into question the value of bioassay following the standard bioassay procedures.

Change of particle size also changed $LC_{50}$ of the products. Decreasing particle size increased $LC_{50}$ (and thus decreased the calculated potency) and increasing particle size decreased $LC_{50}$, thus increasing the calculated potency. Sonication may result in damage of the toxin crystals and thus may cause the decrease in potency, but it is difficult to see how freezing could increase the amount of toxin. Therefore, the changes in $LC_{50}$s in these trials are not regarded to reflect changes in amount of toxin, but to be a function of particle size/distribution.

This relation was confirmed by changing the particle size of various samples. By sonication of primary powders, particle size was reduced and the slope increased (Table 4). Alternatively, by slow freezing fluid samples, particle size increased and slope decreased (Tables 5 and 6). Our results are in agreement with those of Guillet and Duval (1985), who found that intense sample homogenization increased the $LC_{50}$ of various products and the standard IPS82 to mosquito larvae. Guillet and Duval (1985) speculated that the result was an effect of particle size, but this parameter was not measured.

The $LC_{50}$ in a bioassay is a function of concentration and availability of the toxin and the change in particle size must influence the latter parameter since the amount of toxin present was not changed in the trials. By changing particle size, we changed the availability of the toxin to the mosquito larvae.

A simple explanation for the influence of particle size on LC50 would be a preferential uptake of some sizes, but Dahl et al. (1993) did not find that particle size in the range of 0.6–5.8 $\mu$m had a major influence on the chance of ingestion. This is in the lower range of the particle sizes examined here, but Dahl (1988) has also shown that particles up to 100 $\mu$m are ingested.

Sublethal feeding inhibition may also explain the influence of particle size: lepidopteran larvae eating (e.g., *B. thuringiensis kurstaki* with their artificial diet) but not dying within the time of the experiment are stunted and eat less, which is easily observed in the laboratory. A similar sublethal eat-

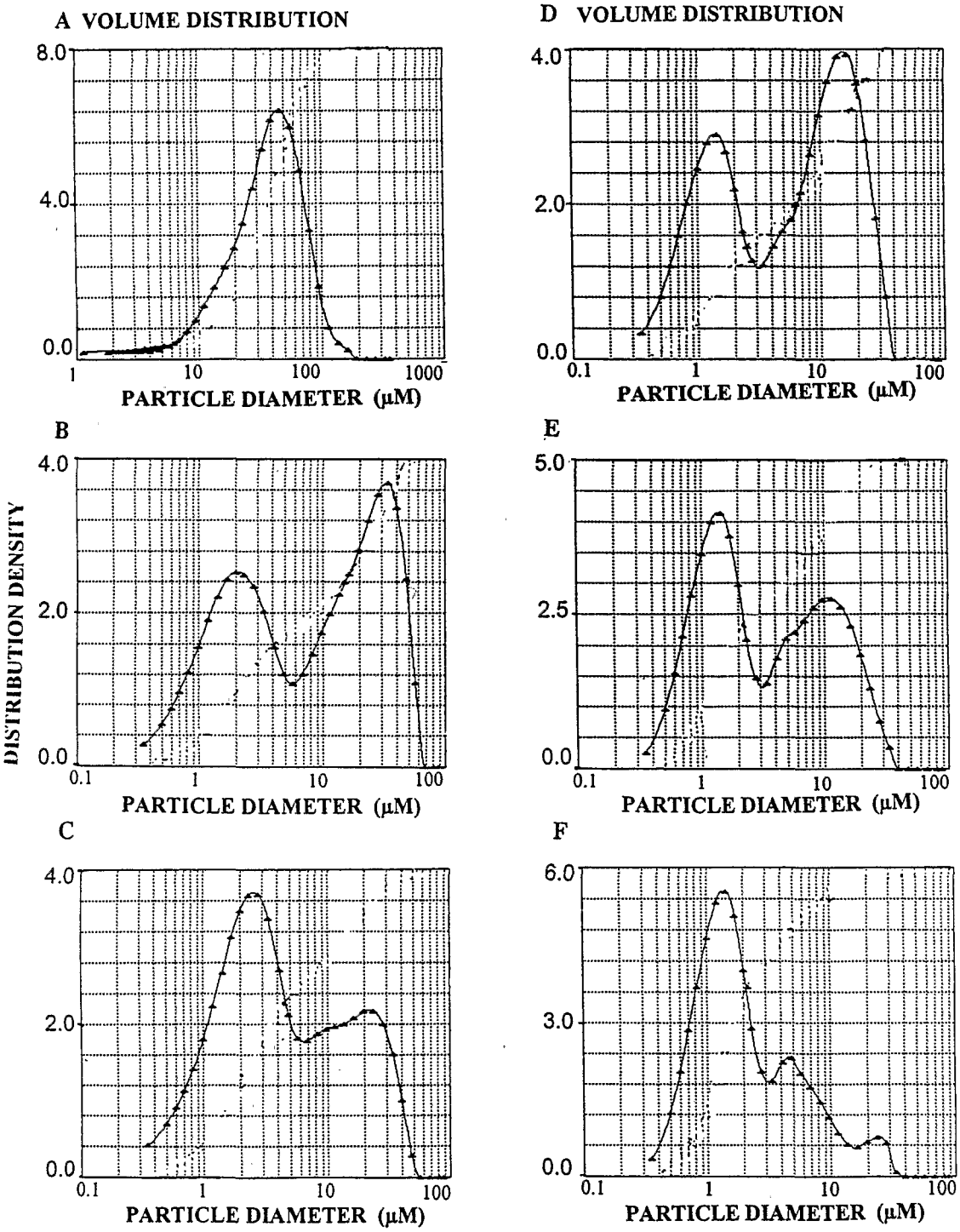


**Fig. 3.** Particle volume size distribution of a *B. thuringiensis israelensis* primary powder (A–C) and of the standard IPS (D–F) after increasing levels of sonication from top to down. Note the change in scale between (A) and (B)–(C). (A) *B. thuringiensis israelensis* primary powder without sonication, (B)sonicated for 120 s in water and (C) sonicated 480 s. Without sonication, the primary powder has a peak at 45 $\mu$m and a median of 37 $\mu$m. (B) Sonication splits up the larger particles and creates 2 groups of particles of reduced size and with a total median of 9 $\mu$m. Further sonication (C) increases the volume of small particles and further reduces the volume of larger particles, median 3.6 $\mu$m. Median values are used in the calculations showed in Table 4. (D) Without sonication, IPS82 consisted of larger and smaller particles. Intense sonication was necessary to split up IPS82 particles (E–F) and thus influence the bioassay, but the intense sonication may also have split up *B. thuringiensis israelensis* crystals in its subfractions, thus influencing the bioassay in an unpredictable way.

**Table 4. Two powder products sonicated to reduce mean particle size and estimated potency**

| Test larvae | Sonication time, s | Median vol, μm | Distribution of particles | Potency ± SE | Slope ± SE |
|---|---|---|---|---|---|
| 2nd Instar | 0 | 37 | 1 peak | 16,600 ± 3,000 | 2.45 ± 0.77 |
| 1st Powder | 120 | 9.0 | 2 peaks | 10,400 ± 900 | 3.20 ± 0.98 |
| | 480 | 3.6 | 2 peaks | 10,800 ± 2,200 | 3.15 ± 0.97 |
| 4th Instar | 0 | 37 | 1 peak | 15,100 ± 1,300 | 2.90 ± 0.49 |
| 1st Powder | 120 | 9.0 | 2 peaks | 12,700 ± 1,900 | 2.84 ± 0.42 |
| | 480 | 3.6 | 2 peaks | 10,000 ± 1,200 | 3.15 ± 0.32 |
| 4th Instar | 0 | 39 ± 1 | 1 peak | 11,800 ± 800 | 3.21 ± 0.21 |
| 2nd Powder | 120 | 2.7 ± 0.4 | 1 peak | 6,700 ± 1,000 | 3.97 ± 0.40 |
| | 480 | 2.0 ± 0.2 | 1 peak | 5,200 ± 1,000 | 4.55 ± 0.92 |

Powders of different origin were used for the tests. The 2nd instar showed to be less resistant to sonication.

ing inhibition may take place for the mosquito larvae. At larger particle sizes, this may prevent mosquito larvae from taking in a lethal dose, provided the dose per particle was big enough to stop the feeding but not big enough to kill them. Sublethal effect would thus be a function of particle size and could explain the dependency of slope and $LC_{50}$ on the particle size. To test this hyphotesis, fine grounded charcoal powder was added to the test beakers after 24 h after reading the assay, and ingestion by the larvae followed. This analysis showed that at all concentrations, nearly all living mosquito larvae ingested particles and therefore could also ingest the *B. thuringiensis israelensis*. Also, many larvae had there stomach full of macerated pieces of their dead fellows, showing that they had been taking in food for a longer period. Obviously, the surviving larvae had not stopped feeding during the assay and there was no sublethal effect in this aspect.

The slope of the dose mortality curve is a function of the heterogenity of the product effect. If product availability is a function of particles sizes, products with broad ranges of particle size distribution will also have low slopes. This is demonstrated by the analysis of Acrobe, a fluid product with a low median particle size, but a broad range of particle size distribution and a low slope. In general, a product with small particles is more homogenously distributed in the water than a product with larger particles and small particles sink slower than bigger, being suspended for a longer time. This could explain the generally higher slopes of the product consisting of mostly small particles compared to those with the larger particles.

**Table 5. Effect of freezing on $LC_{50}$ and slope**

| Sample | $LC_{50}$ index | Slope index |
|---|---|---|
| Sludge | 100 | 100 |
| Frozen sludge | 77 ± 13 | 87 ± 26 |
| Formulated | 100 | 100 |
| Formulated and frozen | 83 ± 8 | 91 ± 6 |

Five formulated and 5 unformulated samples were frozen. The tested products are of different origin and potency. Therefore, indexes are used to indicate direction of change.

Though simple, this theory has a problem: *A. aegypti* larvae also brush the bottoms of the container for particles (Widahl 1988) and should thus ingest the large particles.

**Implications for the Value of Bioassays.** The investigation shows that particle size influences the toxicity of a product as tested in a bioassay on 2nd- and 4th-instar larvae of *A. aegypti*. The smaller the median particle size, the steeper the slope of the concentration-mortality curve. This correlation of particle size, slope and potency may also explain within- and interlaboratory variations as observed in the studies carried out by the WHO Working Group on biological control when they tried to set standards for protocols and potency values of standards. Dulmage's (1980) report to the Working Group stated that the slope of the standard IPS78 was lower than that of the tested fermented beers. Dulmage anticipated that this might have resulted from differences in strain origin of the *B. thuringiensis israelensis* strains used. In the same report, Dulmage tested spray-dried powders from some of these fermentations and obtained a slope similar to that of the standard and significantly lower than of the fermented beers tested on the same days. Because spray-drying increases particle size, our results are in agreement with those of Dulmage (1980).

The potency of IPS80 and of IPS82 was set relative to the older standard IPS78, which by definition was given the potency 1,000 ITU/mg. But analysis of the results from one of the collaborating laboratories showed (Hougard and Coz 1982) that at least IPS80 had a slope significantly higher than that of IPS78 (3.47±.32 versus 2.63±.24). IPS80 was produced in small amounts and quickly used up. Therefore, IPS82 was produced in the same way and from the same strain. They may therefore be assumed to be equal and the slopes of the dose mortality curves are the same (de Barjac and Larget-Thiery 1984). Because the slope of the orginal standard (IPS78) and of the later standards are different, in reality the potency of the 82 standard is obscure. A similar problem does not seem to be related with the USDA *B. thuringiensis israelensis* standard HD-968-S-1983. According to Dulmage

**Table 6. Influence of formulation and freezing on particle size distribution, slope, and estimated potency**

| Sample | Median vol, μm | Slope | Potency |
|---|---|---|---|
| Sludge | 8 | 4.22 ± 0.83 | 1,560 ± 280 |
| Frozen sludge | 40–65 | 2.08 ± 0.60 | 2,850 ± 30 |
| Formulated sludge | 12 | 3.84 ± 0.63 | 1,250 ± 170 |
| Formulated and frozen sludge | 24 | ND | ND |

Particle size distribution shown in Fig. 1F. ND, not determined.

et al. (1985), the slopes of concentration–mortality curves for IPS78 and for this standard were the same.

The implication of our results is that bioassays done with products of different particle sizes compared to that of the standard do not accurately reflect the concentration of *B. thuringiensis israelensis* toxin among products, but does provide valuable information about formulation effect. Potency is not a simple function of the crystal protein concentration but also to a large extent is a function of availability of the toxin. Therefore, although nonbioassay methods may be only roughly correlated to potency as measured in bioassays, they may provide better estimates of the concentration of crystal protein (e.g., Skovmand and Sterndorf 1994). To ensure that the bioassays reflect *B. thuringiensis israelensis* activity, independent of formulation differencies, all products and the standard must be homogenized to the same small particle size (i.e., similar to single cells). This approach was suggested by the the Working Group (WHO Report 1981), but was not followed in the final protocols (de Barjac 1985, McLaughlin 1984). Analyses of the results of the bioassays carried out by the involved laboratories during the work of the Working Group also showed that this suggestion was never followed. The dilemma is of course that the product may then no longer reflect the abilities in the field—if these assays ever did so.

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