BIOASSAY OF FORMULATIONS OF *BACILLUS THURINGIENSIS* FOR USE IN FORESTRY: PANEL DISCUSSION OF THE ROLE OF THE BIOASSAY IN STANDARDIZING FORMULATIONS OF *B. THURINGIENSIS*[1/]

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The panel discussed various aspects of *Bacillus thuringiensis* formulations and fermentations and concluded that the only means at present of standardizing these formulations or discovering more potent strains of the *Bacillus* is through carefully controlled bioassays.

Introduction -- Dulmage

The basic problem in the use of *Bacillus thuringiensis* (*B.t.*) for the control of either the gypsy moth, *Lymantria dispar*, or the spruce budworm, *Choristoneura fumiferana*, is not whether it can work -- it can, as I think that we have seen from the papers that we have heard during these meetings -- but whether it can work reliably. And difficult though it may be to achieve, "reliability" means that we must assure the user that the amount of activity present in a *B.t.* formulation is what the label says it is -- each and every time. This means that the quantity of delta-endotoxin in a formulation (i.e., the potency of a formulation) must always be the same, and that the remainder of the formulation will be assembled in such a way that the efficacy of the formulation will repeat from application to application.

Potency and efficacy depend on each other -- how much delta-endotoxin a formulation contains and how effectively a formulation performs are, and always will be, interdependent. If we restrict ourselves to a specific number of IU's/mg or ml in a series of formulations, the effectiveness of the formulations will then depend on the different qualities of the formulation ingredients. This concept frequently gets lost in our consideration of *B.t.* (or, for that matter, any microbial insecticide). Most people are able to accept the concept that changes in composition of the carriers in chemical formulations can make formulations containing the same quantities of active chemical ingredients (say a synthetic pyrethroid) more or less effective in field applications. In the case of chemical insecticides, we realize that, to define a formulation, we need to know both the level of active ingredients and the recommended application rates of formulations of chemical insecticides like the synthetic pyrethroids. We accept this and agree that both are needed on the label. The same applies to a *B.t.* formulation. Just as in the case of a chemical insecticide, we also need to know both the level of active ingredients (IU/mg or ml) of the delta-endotoxins produced by *B.t.*, and we need to know the recommended application rates of the *B.t.* formulations. Here too, both are needed on the label. I don't know why, but what seems clear and well understood and accepted in the case of chemical-based insecticides, is not clear, understood, or accepted in the case of *B.t.* formulations. In the case of *B. thuringiensis*, if formulations produced by two companies contain the same levels of delta-endotoxin as measured in bioassays, but have different effectiveness in the field so that the recommended application rates are different, then it is frequently claimed that the bioassays that determined the IU's/mg or ml of the *B.t.* put into the formulation must be wrong. It is then concluded that biological materials such as *B.t.* cannot be standardized.

This conclusion is not new -- it arose in the early days of the commercial production of *B.t.* Because of it, companies and scientists predicated much of their work on the belief that "there is no reliable bioassay of *B.t.*" As a result, each company producing *B.t.* developed its own method of standardizing its product -- methods ranging from spore counts, to microscopic crystal counts, to bioassays against this insect species or that, to, I sometimes used to think, assessing which planet was in which sign of the zodiac at the time the formulation was prepared. This was wrong then -- and it is wrong now. Formulations of *B.t.* can be standardized, and once the effectiveness of a given composition of carriers and IU's has been determined, formulations can be made reproducible by assaying the quantity of delta-endotoxin contained in the formulation and keeping it constant. The user can rely on the IU's determined in a properly designed bioassay. The design of the bioassay -- and this will be covered in detail by Dr. Beegle, may have to be adapted to the situation -- certainly, Dr. Beegle and industry in a recent -- and I think outstanding -- cooperative study have greatly improved our bioassay procedures and our understanding of these procedures. Thanks to their work bioassays of even the newer flowable formulations are reliable, the IU's expressed on the label are accurate, and the efficacy of the product can be trusted. We have suffered in the past from the practice of each company designing its own bioassays and procedures. This led to confusion (and really to chaos) to the user, and,

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as the user tried to follow IU's determined in different ways, he lost faith, not only in the formulation that he was using, but in B.t. itself.

"Trust" is not meant to imply that any one company might intentionally market an inferior product, disguising it with faulty assays. But, as will be seen, improper design of bioassays can lead to misleading potency determinations. There has to be some agreement between producers, the USDA (ARS and FS), and the government regulatory agencies as to what we are measuring and how we should measure it. We must not let the competitive urge lead us to forget how important the standardization of bioassay procedures between producers is. This point should be reiterated: Let each company go its own way and put its own IU's on its labels, and we are going to so confuse the user that his trust in B.t. will be destroyed.

Fortunately, the producers of B.t. realize this, and industry and the USDA, under the leadership of Dr. Beegle, has been exploring improved ways to standardize B.t. taking into account the various problems that have arisen in the standardization process, and Dr. Beegle will now discuss these problems and what is being done to coordinate bioassays.

Problems in Standardization -- Beegle

Formulations of B.t. are standardized in bioassays against a selected test insect. In these assays, the test insect is exposed to diet containing variable levels of the B.t. formulation and, after a suitable period of incubation, the percentage kill for each level is recorded, and the LC_{50} of the formulation is determined. The LC_{50} of the test formulation and the LC_{50} of a standard reference material tested at the same time against the same population of insects are compared. Since the IU/mg of the standard is known, the potency of the formulation can be determined by the following formula:

Potency, IU/mg, test sample =

$$\frac{LC_{50}\ \text{Standard} \times \text{Potency Standard, IU/mg}}{LC_{50}\ \text{Sample}}$$

In 1971, representatives of the three commercial producers of B. thuringiensis in the United States and the USDA met in Brownsville, Texas, and agreed in principle to a standardized bioassay procedure using larvae of the cabbage looper, Trichoplusia ni, to standardize the potencies of their respective products (Dulmage et al. 1971). Because of the adoption by industry in 1970 of a much more potent strain of B. thuringiensis, which was of a different serotype than earlier B.t.'s, the Pesticide Regulation Division of the U.S. Environmental Protection Agency requested that a standard be produced and adopted which was the same serotype as that being commercially produced (subsp. kurstaki). This was accomplished in 1972, when HD-1-S-1971 was adopted as the Primary U.S. Reference Standard with an assigned potency of 18,000 IU//mg (Dulmage 1973). Recently, the supply of HD-1-S-1971 became exhausted, and a new standard, labeled HD-1-S-1980, has been prepared and assigned a potency of 16,000 IU/mg after comparison with HD-1-S-1971 and E-61. Today all preparations of B. thuringiensis produced in the U.S. for use against lepidopterous larvae are standardized against either HD-1-S-1980, or an internal HD-1 standard which was initially standardized against HD-1-S-1971.

In 1980, a problem emerged in the standardization of lepidopterous-active B. thuringiensis preparations. The Brownsville USDA laboratory was contracted by the U.S. Forest Service to bioassay the tank mixes of the flowable B. thuringiensis products, Thuricide and Dipel, used in the CANUSA B. thuringiensis Core Tests. The potencies determined by Brownsville, using neonate T. ni larvae and diet containing antibiotic, were only 40-60% of the label values. Both commercial products were standardized using 4-day-old T. ni larvae and diet without antibiotic. In 1981, research was undertaken by the USDA and the Upjohn Co. to determine the reason for the difference. In these studies two preparations of B.t. from the Upjohn Co. (SOK W and SOK L) and two formulations from Abbott Laboratories (Dipel WP) were tested. The results are shown in Table 1. In the case of the SOK wettable powder (W) and liquid (L), it is clear that the use of 4-day-old larvae in the bioassay results in higher determined potencies than when neonate larvae are used. In the case of the Dipel wettable powders (WP), one of them (0452BJ) was larval age sensitive and the other (F-1980) was not. In my experience that is typical of Dipel products -- some are larval age sensitive and others are not.

In addition, the USDA and the Forest Service set up a blind test where differing amounts of viable HD-1-S-1980 were added to Sandoz and Abbott flowable carriers. These samples, along with flowable Dipel and Thuricide samples were coded and sent out for assay to the USDA laboratories in Brownsville, Texas, and Columbia, Missouri, and to Abbott and Sandoz. The results of these assays are presented in Table 2. Except for two values (2870 for Thuricide, and 16,100 for Dipel, the results in Table 2 for the commercial flowables substantiates the results observed in Table 1 concerning the commercial flowables: the use of 4-day-old larvae in the bioassay resulted in significantly higher potencies than when neonate larvae are used. It was shown by Beegle et al. (1981) that 1-day-old T. ni larvae respond the same as neonate larvae in B. thuringiensis bioassays. It is noteworthy that larval age did not significantly affect the potencies of the samples where HD-1-S-1980 was incorporated into the commercial flowable carriers. This indicates that the observed larval-age-difference effect on determined potencies is due to differences in the bacteria rather than in the formulation. In fact, Yamamoto (pers. comm.) has cautioned that anyone who uses the HD-1 isolate

Table 1. Effect of Larval Age and Antibiotic on Potency Determinations of Commercial *Bacillus thuringiensis* Products.[a/]

Preparation	*Trichoplusia ni* Larval Age	Anti-biotic in Diet?	USDA Brownsville No. Assay	USDA Brownsville $\bar{X}$ IU/mg or $1X10^3$/ml	USDA Brownsville ± SD	USDA Brownsville % CV of Assays	Upjohn No. Assays	Upjohn $\bar{X}$ IU/mg or $1X10^3$/ml	Upjohn ± SD	Upjohn % CV of Assays
SOK W 747 JF	neonate	yes	3	13,600	± 814	6	5	11,600	±1910	16
SOK W 747 JF	neonate	no	3	15,400	±1510	10	6	13,400	±2040	15
SOK W 747 JF	4-day-old	yes	3	27,700	±5300	19		-		
SOK W 747 JF	4-day-old	no	4	30,100	±5370	18	7	36,300	±6930	19
SOK L 645 JB	neonate	yes	6	3,640	± 692	19	8	2,950	± 514	17
SOK L 645 JB	neonate	no	3	3,640	± 369	10	4	3,700	± 534	14
SOK L 645 JB	4-day-old	yes	3	6,310	± 936	15		-		
SOK L 645 JB	4-day-old	no	5	7,850	±1310	17	6	9,420	±1260	13
Dipel WP 04523BJ	neonate	yes		-			9	12,300	±1670	14
Dipel WP 04523BJ	neonate	no		-			2	12,100	±1310	11
Dipel WP 04523BJ	4-day-old	no		-			3	24,300	±1580	6
Dipel WP F-1980	neonate	yes	3	14,800	±2280	15		-		
Dipel WP F-1980	neonate	no	4	16,300	± 594	4		-		
Dipel WP F-1980	4-day-old	yes	3	12,000	±2490	20		-		
Dipel WP F-1980	4-day-old	no	4	11,500	± 675	6		-		

[a/] Taken from Beegle et al. (in press).

Table 2. Results of Blind USDA-Industry Bioassay Test.[a/]

Laboratory	Larval Age	No. Assays	Range of CVs	IU/mg 01-S 1980	02-S 1980	03 Thuricide	04-A 1980	05 Dipel	06-A 1980
USDA-Brownsville	neonate	18-20	15-18	1700	2610	1290	4170	5630	5620
USDA-Columbia	1-day-old	3- 4	-	1660	2010	1380	4210	16,100	6620
Sandoz	4-day-old	-	9-19	1960	2760	3970	3310	8110	4520
Abbott	neonate	6-14	5-15	2340	2870	2870	3180	3720	3480
Boyce Thompson Calculated	-	-	-	1720	2570	3430	3810	8800	5800

[a/] Lewis, F.B., Report on Blind BT Sample Bioassays, USDA-Forest Service, New Hamden, CT, 1982.

to study the genetics or chemistry of *B. thuringiensis* should keep in mind that there are differences in the plasmid and P-1 HPLC patterns among cultures labeled as HD-1.

Because of the discovery that the use of a standard does not correct for differences in assay procedures in all cases, the USDA and the U.S. producers and/or marketers of *B. thuringiensis* products held a meeting in 1982 and decided that we needed a new standardized lepidopterous *B. thuringiensis* bioassay that everybody would follow. The following summarizes the decisions reached at that meeting:

1. Rearing

   Origin of *T. ni* colony, rearing diet, rearing conditions, and rearing techniques and procedures will not be standardized.

2. Bioassay

   a. Diet - Brownsville diet without antibiotics or KOH.
   b. Diluent - deionized or distilled water.
   c. Sample homogenization - sonicate in bath sonicator for 5 min. at sonicator loading of 3 ml (container size)/watt.
   d. Number of dilutions - enough for a minimun of five on-curve dosage mortality points, at least two above and two below LC_{50}.
   e. Type of dilutions - 3:4 (75%).
   f. Diet incorporation - 1:10 with 55°C diet, mix for 2 min.
   g. Number of insects/dose - minimum 25.
   h. Age of larvae - 4-days-old at 30°C.
   i. Holding (infesting) density - multiply or singly.
   j. Holding time and temperature - 4 days at 30°C.

3. Potency Determinations

   a. Each preparation must be bioassayed at least three times over at least two days (three days preferable).

b. A mean potency value for a preparation should consist of at least three potency values from valid assays whose group coefficient of variation is less than 20%.

c. Valid assays are defined as those with a minimum of five on-curve points, with at least two above and two below the LC_{50}, significant regressions, significant F values, and 95% confidence values of less than a 3X range. Assays with atypical slopes, significantly high X^2s, erratic points, or 95% confidence value ranges greater 2X, but less than 3X, should be discriminated against if necessary, but not be considered invalid.

Table 3 is presented to establish the validity of the determined potency of 16,000 IU/mg for the new U.S. B. thuringiensis HD-1 standard. E-61, which had an assigned potency of 1,000 IU/mg; HD-1-S-1971, which had an assigned potency of 18,000 IU/mg; and HD-1-S-1980 were concurrently bioassayed by four companies and USDA-Brownsville. On the basis of over 200 bioassays, it was determined that HD-1-S-1980 had a potency of 16,000 IU/mg. We have known for years that HD-1-S-1971 was not homologous with "normal" HD-1's in its activity towards larvae of Heliothis virescens. Since HD-1-S-1971 is a little over twice as active toward H. virescens larvae than most HD-1 preparations, the Tn/Hv ratio (the ratio will be discussed in the next section, but represents the comparison of potencies determined against T. ni and those determined against H. virescens) of most HD-1 preparations is a little over 2. HD-1-S-1980 appears to be homologous in this respect to most HD-1 preparations, as the mean Tn/Hv ratio of 87 HD-1 preparations assayed against this standard at Brownsville was 1.04.

Table 3. Standardization of HD-1-S-1980.

Sample	Standard	IU±SD	CV	No. Assays
Abbott Laboratories				
E-61	HD-1-S-1971	853± 150	17	14
E-61	HD-1-S-1980	827± 145	17	14
HD-1-S-1971	E-61	21,800±4390	20	14
HD-1-S-1971	HD-1-S-1980	17,500±1560	9	14
HD-1-S-1980	E-61	19,900±3640	18	14
HD-1-S-1980	HD-1-S-1971	16,000±2770	17	64
Biochem Products				
E-61	HD-1-S-1971	758± 191	25	9
E-61	HD-1-S-1980	683± 342	50	9
HD-1-S-1971	E-61	24,600±4600	19	9
HD-1-S-1971	HD-1-S-1980	16,000±4090	26	9
HD-1-S-1980	E-61	25,700±6180	24	9
HD-1-S-1980	HD-1-S-1971	19,000±4390	23	9
Sandoz, Inc.				
TS[a]	E-61	21,500±2040	4	13
HD-1-S-1971	E-61	16,600±5820	15	8
HD-1-S-1971	TS	15,900±5760	17	12
HD-1-S-1980	E-61	14,200±2440	7	7
HD-1-S-1980	TS	14,900±3230	11	36
HD-1-S-1980	HD-1-S-1971	14,700±4030	11	6
Upjohn Co.				
E-61	HD-1-S-1971	1,135± 381	33	3
E-61	HD-1-S-1980	1,319± 260	20	3
HD-1-S-1971	E-61	17,000±4950	29	3
HD-1-S-1971	HD-1-S-1980	18,000±3592	20	8
HD-1-S-1980	E-61	12,400±2120	18	3
HD-1-S-1980	HD-1-S-1971	16,500±3070	19	8
USDA Brownsville				
E-61	HD-1-S-1980	1,280± 247	19	32
HD-1-S-1971	HD-1-S-1980	21,300±4170	20	42
HD-1-S-1980	E-61	12,900±2570	20	30
HD-1-S-1980	HD-1-S-1971	15,700±1990	13	23
All Sources Combined: Nonweighted				
E-61		980± 260	27	84
HD-1-S-1971		18,700±3070	16	119
HD-1-S-1980		16,500±3800	23	209
All Sources Combined: Weighted[b]				
E-61		1,010± 392	39	84
HD-1-1971		19,600±4360	22	119
HD-1-S-1980		16,000±3139	20	209

[a] Sandoz internal standard.
[b] Weighted as to number of assays.

Reproducibility and the Activity Ratio -- Dulmage

One of the most important and fascinating aspects of the delta-endotoxins produced by B. thuringiensis is that different subspecies -- and, indeed, isolates -- of this Bacillus species, kill different insects or differ in their relative activities against them. Dr. Beegle has discussed the relative differences found between different isolates of subsp. kurstaki in their activities against T. ni and H. virescens. This can lead to two legitimate concerns: (1) Will the same endotoxin be produced in repeat fermentations, or will the type of endotoxin vary? (2) Can we use one insect species in our bioassays -- and does that have to be the target insect? All this, of course, leads to the label and what influence all this has on what is put on the label.

These are important questions and pose serious problems. Table 4 illustrates the differences between subspecies and isolates. The table shows the spectra of insecticidal activity of a series of different B.t.'s produced by subsp. alesti, kurstaki (k-1), kurstaki (k-73),

galleriae, and aizawai. As the table shows, there is a wide difference between them in their activities towards lepidoptera and in their relative activities against different insect species when they are active against them. (One variety, not included in this table, is not even active against lepidopterous insects, although it is extremely active against mosquitoes and aquatic black flies.) With this proliferation of different spectra, the problem of standardization seems almost insurmountable. Industry, Dr. Beegle, and myself all agree on the desirability of using T. ni as the assay insect of choice for the present commercial delta-endotoxins active against lepidopterous insects. Yet, as the table shows, the existence of delta-endotoxins with different spectra of insecticidal activities makes it perfectly possible that the potency of delta-endotoxins produced by two isolates of B.t. may be the same when measured against T. ni but may differ considerably when evaluated against L. dispar. However, if the same type endotoxin is produced in replicate fermentations of each of these isolates of B. thuringiensis, then our problem merely parallels the case with different formulations of chemical insecticides, where the same quantities of a chemical in different formulations may differ widely in their effectiveness in control of a target insect. In the case of the chemicals, the differences arise from differences in their formulation. Microbial formulations may vary for this reason too, but with microbials, the differences may also reflect differences in the chemical structure of the delta-endotoxins produced by different isolates, i.e., the basic activity of the endotoxin. With our present knowledge, we do not know what these differences are -- remember, we cannot weigh or identify in a quantitative chemical assay the delta-endotoxins that we are producing -- we only measure their activities: i.e., what insects they kill and how well they kill them.

Table 4. Spectra of Activities of Selected Subspecies of Bacillus thuringiensis.

Culture No.	Variety	Crystal Type	Potency - IU/mg Measured vs.: T. ni	H. vir.	H. cunea	B. mori
HD-1	kurstaki	k-1	39800	15400	47200	50500
HD-73	kurstaki	k-73	23100	34500	12800	Inact
HD-263	kurstaki	k-1	39600	54600	18100	18400
HD-70	alesti	ale	Inact	Inact	47900	34000
HD-160	galleriae	gal	5920	894	23800	4100
HD-275	aizawai	aiz	8110	969	19500	44400

To illustrate the problem, let us create a hypothetical case of two formulations of B.t., each containing, say, 10,000 IU/mg of activity as measured against T. ni. However, one is several times more active against L. dispar than the other. How do we standardize formulations of each for use against this insect? Must we measure each and every formulation only against every target insect that we desire to kill? Such a path will lead to chaos and confusion -- and very likely to the need of a multiplicity of labels for each B.t. formulation. Fortunately, there seems to be an alternative.

Potencies of formulations of B.t. delta-endotoxins are determined relative to a selected reference standard, as Dr. Beegle has discussed. If the formulation being tested contains the identical endotoxin to the standard, then it will not matter which insect species is used in the bioassay. However, if the test endotoxin and the standard endotoxin are not homologous -- i.e., if they differ in their spectra of activity -- then the choice of insect will have a profound influence on the IU's determined. For example, a formulation of subsp. alesti would be considered inactive if bioassayed against T. ni, but would be considered very active if assayed against the webworm, Hyphantria cunea. In a less extreme example, a formulation of subsp. kurstaki (k-73) might have a potency of 20,000 IU/mg if measured against T. ni, but have a potency of 40,000 IU/mg if measured against H. virescens. By constructing a fraction from these data, we can derive an "Activity Ratio." An activity ratio of a formulation is simply a straight-forward comparison of the activity (in IU's) against two different insect species. Thus in our example here, the Tn/Hv activity ratio is simply determined by dividing the activity determined against T. ni by the activity found against H. virescens, as shown in the following equation:

$$\text{Tn/Hv Activity Ratio} = \frac{20{,}000 \text{ IU/mg}}{40{,}000 \text{ IU/mg}} = 0.500$$

The ratio will, of course, vary with the endotoxin being tested and with the insect species being used. The value of the ratio thus becomes an accurate mathematical definition of a portion of the spectrum of activity of a delta-endotoxin.

Encouragingly, our data so far leads to the conclusion that, while the spectra of activity of the delta-endotoxins produced by different isolates may differ widely, the spectra of activity of the delta-endotoxins produced under different fermentation conditions by a single isolate of B.t. are reproducible. This is shown for four formulations in Table 5. If this is true -- and we believe that it is -- then, if we know the relative activities of an endotoxin against T. ni and the gypsy moth, we can assay against the cabbage looper and reliably predict the potency of the endotoxin against the gypsy moth. Once we are satisfied that this is so, then the "active ingredients" of B.t. on a label of a forestry formulation can be safely listed as IU/mg vs. T. ni and represent how much B.t. was put into the formulation, and the efficacy of the formulation can be expressed in pints or pounds/acre. Activity and efficacy are being presented on the label in the same way as a chemical -- and, once we have gotten used to this, it should be no more of a communication than with chemicals.

Table 5. Reproducibility of Activity Ratios.

Culture No.	Number of Formulations	Avg. Tn/Hv Ratio	Coefficient of Variation
HD-1	60	2.14	0.25
HD-241	26	0.63	0.24
HD-244	26	1.54	0.23
HD-263	136	0.44	0.29

Thus it is my view that an assay insect does not need to be the target insect, but need only be a convenient, easy-to-rear-and-use insect which is susceptible to the delta-endotoxin in the product. The cabbage looper seems to fit these characteristics. However, it may very well happen that someone will discover a new B.t. that is very effective against L. dispar that may not be active enough against T. ni for this insect to serve as an assay tool. In such a case, of course, another insect species may be needed. However, at present no such B.t. is known, and T. ni should be satisfactory for these assays. The label should then read in IU's as determined against this insect. The differences in efficacy that may occur should be shown in the recommended application rates, not as different IU's. It has been suggested that one could include on the label a Tn/Ld Activity Ratio as a parenthetical addition. However, this may be confusing and of little value to the user. Thus it is our belief that the bioassay against T. ni should remain the key for standardizing B.t. in forestry formulations.

Miscellaneous Aspects of Evaluating New Isolates of B.t. -- Dubois

In a paper that I presented earlier in this meeting, I discussed some of our research seeking new isolates of B.t. with increased activity over HD-1 against L. dispar. Drs. Dulmage and Beegle have just discussed their work with bioassays and activity ratios, particularly as they relate to T. ni and H. virescens. In this portion of our panel program, I would like to discuss how some of their observations relate to our experience with L. dispar.

Tn/Hv Ratios

A few years ago, our FS laboratories at Hamden, CT, joined in an International Screening Program to survey the spectra of insecticidal activities of about 350 isolates of B. thuringiensis. The results of this survey were very complex and will not be discussed here (for more details, see Dulmage et al. 1981). One of the observations that was reported was that the Tn/Hv activity ratios of formulations derived from isolates of subsp. kurstaki and which contained the k-1-type crystal were about 2.0. Formulations of this subspecies which contained the k-73-type had Tn/Hv ratios of about 0.40. The difference in the two types seemed to lie in the activity of k-73 formulations against H. virescens. Simply, the k-73 endotoxin was more active against H. virescens than was the k-1 toxin. The parallel to L. dispar was interesting. When we examined the more potent isolates of B. thuringiensis, we found that these formulations had Tn/Hv ratios of about 2.0, belonged to subsp. kurstaki, and contained the k-1 crystals. Interestingly, those isolates of subsp. kurstaki that produced k-73-type crystals were not as active vs. L. dispar. These generalities should not be taken to mean that all isolates of a given subspecies will have the same potency. Some formulations will be very active, some will have little or no activity, and the activities of others will lie somewhere in between.

Reproducibility of the Response of L. dispar to B.t.

The primary need for an accurate B.t. bioassay is, of course, a supply of uniform, healthy, bioassay insects. Even with such an insect, however, the assay will not be reliable if the response of the insect to B.t. formulation changes according to the source of the insect. To see if this were a serious problem in assays with L. dispar, we have compared the response of a series of sources of L. dispar to the two standard B.t. formulations, HD-1-S-1971 and HD-1-S-1980. The cultures of L. dispar studied were diverse and included the New Jersey strains in our laboratory and the F_{18} through F_{24} generations of our laboratory colony. The responses of L. dispar to these standards were evaluated by the LC_{50}'s determined for the standard against the various insect cultures. The results of these assays are summarized in Table 6. The responses were very uniform, and what variations were observed seemed to be due to the diet, not to any change in the response of the insects. I think that we have reasonable assurance that the source of the L. dispar does not affect the response to B.t. in our bioassays.

These tests were all run with laboratory-reared insects. They leave open a very important question -- How do the responses of laboratory insects compare with wild insects? -- i.e., do we need to maintain a laboratory colony generation after generation, or could we collect wild egg masses in the field each year and use these for our bioassays? The study is not yet completed, but we have examined the responses of insects reared from egg masses of wild sources of L. dispar collected in Connecticut, New Hampshire, New York, Massachusetts, and Pennsylvania. We found some minor differences in the responses of the different insect strains (which might very well have been due to diet again), but overall, the LC_{50}'s determined for the standard formulations did not vary much with insects of different origin. This is not to say that wild insects are the insect of choice -- one must consider the risk of results being distorted by the presence of unrecognized disease in the wild culture.

Table 6. Slopes and LC_{50} of the Bacillus thuringiensis Standards, HD-1-S-1971 and HD-1-S-1980, Bioassayed Against the New Jersey F_{18} to F_{24} Generations of Lymantria dispar L. Continuously Reared on Artificial Diet.

Fermentation	No. of Bioassays	Slope	C.V.	LC_{50} (µg/ml)	C.V.
		HD-1-S-1971 (18,000 IU/mg)			
F_{18} (1979-1980)	4	4.25	0.16	7.82a[a/]	0.09
F_{19} (1980)	4	4.54	0.20	5.83ab	0.27
F_{21} (1980)	28	4.72	0.20	5.70ab	0.09
F_{22} (1981)	5	4.86	0.27	7.38ab	0.38
		HD-1-S-1980 (16,000 IU/mg)			
F_{22} (1981)	24	4.62	0.21	5.33ab	0.33
F_{23} (1982)	29	4.87	0.21	5.01ab	0.25
F_{24} (1983)	13	5.09	0.16	5.24ab	0.25

[a/] Numbers followed by the same letter are not significantly different from each other at the 5% level (Scheffe's method for all possible contrasts).

Table 7. LC_{50} of Bioassays of Several Sources of F_1 Generations of Instar II Lymantria dispar L. fed on Fresh and on Old Diet.

Larvel Source	Diet	LC_{50} (µg/ml of diet) on Dates: 2/9	3/13	3/14	3/24	3/28	3/31
New Jersey	fresh	6.9			5.9	5.0	6.8
F_{26}	old		17.8	13.0			
Cockaponset, CT	fresh	12.4			---	---	8.3
F_1	old		13.6	21.6			
Storrs, CT	fresh	7.9			7.9	6.9	---
F_1	old		15.3	27.3			
New Hampshire	fresh	8.4			6.3	6.4	---
F_1	old		13.1	16.0			
Mashpee, MA	fresh	8.3			6.9	7.4	---
F_1	old		15.7	20.6			
Pennsylvania	fresh	10.6			6.7	7.9	
F_1	old		9.2	34.6			

Influence of Diet on the LC_{50}

The composition of the diet can affect the observed LC_{50}'s of B.t. formulations. This was discovered early in our program. We began then, and still do today, to buy a premixed insect diet from a single supplier. Over a long period of time, we amassed considerable experience in just where the LC_{50}'s of our standard formulations and test samples would be. We recently received a new shipment of diet that was mistakenly contaminated with antibiotics. When we attempted to use this diet, we found that the LC_{50}'s of both the standard and the test samples were very high -- it appeared that our materials had lost activity. When we used antibiotic-free diet that had been stored frozen for 18 months, the LC_{50}'s were again very high, indicating a loss of activity of the B. thuringiensis preparations, an increased resistance of the insect, or an incompatability to the diet. Meanwhile, we received a new batch of diet, and the results of our assays dropped, returning to the "normal" range (Table 7). Thus the cause of the erratic LC_{50}'s lay, not in the insect, but in the diet.

Influence of Formulation on the Bioassay

Earlier, Dr. Beegle described some of the problems that they encountered with bioassays of flowable formulations and the influence of insect age and the presence or absence of antibiotic in the diet. We have had similar problems. During the field work last year in Maine, we used drums of Thuricide 32LV and SAN 415 32LV. We sent samples to Donald Hostetter in Columbia, Missouri, for him to assay by his surface contamination technique against T. ni (different from the usual T. ni assays), while we assayed duplicate samples against L. dispar. The assays obtained were very similar to each other and to the results obtained the previous year at Brownsville against T. ni.

This led us to believe that the causative mechanism for the discrepancies observed in the bioassays against L. dispar was the changes in the diet and larval age in the various assays. However, the composition or structure of the delta-endotoxins in the various B.t. isolates may also play a role. This observation may be reinforced by the assay results obtained with two

experimental flowable concentrates prepared by Sandoz for a comparative study of HD-1 and NRD-12 (HD-945). When the concentrates of the two strains were bioassayed against T. ni, both were reported by Sandoz to contain 32 BIU/gal, but by Dr. Hostetter's assays against T. ni were found to be only 14 BIU/gal -- a discrepancy vs. the label potency similar to that found in our previous assays of commercial concentrates. However, when these two flowables were bioassayed against L. dispar, the HD-1 concentrate assayed 15 BIU/gal (similar to the assays vs. T. ni), while the NRD-12 concentrate assayed 42 BIU/gal (nearly 3 times higher than the HD-1 concentrate with an "R-value" of 2.3). Similar differential assays were found in the tank mixes prepared from these concentrates: Assays against the HD-1 mix were essentially the same regardless of which insect was used in the assay; NRD-12 again assayed nearly 3 times higher against L. dispar (R-value = 2.9) (Table 8). Thus the two materials were similar when measured against T. ni, but different when measured against L. dispar -- further evidence of the importance of Dulmage's warning about the necessity for monitoring activity ratios in these studies.

Table 8. Differences Between the Potency Estimates of Thuricide 32LV (HD-1) and SAN 415 32LV (NRD-12) and their Diluted Tank Mixes[a/] when using Lymantria dispar and Trichoplusia ni.[b/]

Source of Concentrate	Potency Estimates in BIU/gal: Lymantria dispar	R[c/]	Trichoplusia ni	R
Concentrate: labeled 32 BIU/gal				
Thuricide 32LV	18.1		13.7	
		2.3		1.1
SAN 415 32LV	42.2		15.3	
Tank mixes: calculated 24 BIU/gal				
Thuricide 32LV	5.4		9.5	
		2.9		1.2
SAN 415 32LV	15.8		11.4	

a/ Samples were from the spruce budworm spray test in Maine, 1983. Tank mixes are from the concentrates diluted with water.

b/ T. ni assays done by D. Hostetter, USDA, ARS, Columbia, MO.

c/ $R = \frac{LC_{50}\ NRD\text{-}12}{\text{Potency HD 1}}$

Summary

The results of the work discussed in these papers, combined with the results previously reported in the literature, show very strong evidence that, at our present stage of knowledge, it is essential that we rely on a bioassay as our only means of standardizing products formulated from B. thuringiensis fermentations. The results also indicate the need for a standard in the evaluation of these bioassays, and finally, show the value of activity ratios as means of discriminating between the delta-endotoxins produced by the various isolates of B. thuringiensis. The reproducibility of these activity ratios, in particular the relative activities of L. dispar vs. T. ni, confirms that one insect species (e.g., T. ni) can be used to standardize a formulation against another insect species (e.g., L. dispar) with confidence that the relative activities will extrapolate between insect species.

Joint Statement by Panel Members -- Dulmage, Beegle, Dubois

We find no fault with the use of T. ni as an assay insect for standardizing commercial formulations of B. thuringiensis (although care must be taken to develop a proper protocol for these assays, as shown by Beegle in his talk), and, since its use is so widely accepted, we recommend this insect for these assays. We also recommend the use of HD-1-S-1980 as a standard in these assays. We disagree with the use of spore counts as a substitute for these bioassays, since we see no correlation at all between spore counts and insecticidal activity. The spore count may serve a purpose in following the immediate dispersal of a spray in test application, but not in following the potency or persistence of the B. thuringiensis formulations.

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Summary

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The theme running through all sessions of the symposium was that B.t. is an effective and reliable material for use in management of the gypsy moth and spruce budworm. This new status is the result of recent developments in the use of increased dosages, finer droplet deposition, ULV application rates, and competitive costs.

The symposium reaffirmed the outcome of several recent conferences on microbials by identifying two major areas in which continued international cooperative research and development is needed; (1) molecular biology and modification to develop more virulent strains of the pathogens; and, (2) more reliable formulation, application, and deposition technology.