Toxicology of the Microbial Insecticide, Thuricide

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One of the advantages of the new living insecticide based on the viable spores of the microorganism *Bacillus thuringiensis* Berliner is its nontoxic nature for man, other animals, and plants. This characteristic was firmly established in a series of tests which included an unusual human volunteer test. The toxicology studies described in this paper represented a pioneering effort, as there was no precedent to guide the manufacturer or government officials in establishing that the proposed use of the pesticide would be without hazard to health.

THURICIDE (Bioferm Corp., Wasco, Calif.) is a live pest control agent which is a fatal, quick-acting disease for susceptible insects. The active principle consists of live spores of the microorganism *Bacillus thuringiensis* Berliner, a bacterium first isolated in 1911 from diseased larvae of the Mediterranean flour moth. A review of the literature (*2, 12–18*) reveals no authenticated instance of this true insect pathogen having caused an infectious disease in warm-blooded animals or plants either experimentally or in nature. On December 10, 1958, the Food and Drug Administration authorized the application of the microbial insecticide Thuricide directly to food and forage crops under the conditions of a temporary exemption from a tolerance. Part of the exhaustive 2-year toxicological study which satisfied government officials and the manufacturer as to the safety of Thuricide is described.

Experimental

The toxicological studies on Thuricide were divided into four groups: infectivity and sensitization, acute and chronic toxicity, effect on human volunteers, and toxicity in the field. Some of the tests in the first, second, and third groups are presented here.

The Thuricide used in most of the tests contained approximately 9×10^9 viable spores of *B. thuringiensis* per gram, and in the human volunteer study, 3×10^9 viable spores per gram. Spore count was determined by a plating method (*3*). In addition to the active spores, the product contained a diatomaceous earth filler which had some toxic effect on animals in the massive doses given. When vegetative or sporulated cultures of the microorganism itself were used in these tests, they were designated as *Bacillus thuringiensis* Berliner.

Virulence of Thuricide Following Serial Passage through Mice. One half gram of Thuricide was added to 20 ml. of nutrient broth and incubated at 37° C. for 72 hours. Immediately before injection into the test animals, another 0.5 gram of the sample was added to the incubated material, providing both spores and vegetative forms of the microorganism.

Thirty-five white mice, weighing 17.0 to 23.0 grams, were assembled into seven groups of five mice each. The animals were placed in wire cages and fed a standard mouse diet and water ad libitum. The mice in group 1 were injected intraperitoneally with 1.0 ml. of the prepared material. After 6 hours, 0.3 ml. of blood was withdrawn by cardiac puncture and injected intraperitoneally into group 2. This technique was repeated for a total of six transfers using five more groups of mice. Group 7 was weighed prior to injection and after 24 and 48 hours. No weight losses nor abnormal symptoms during the test period were observed. In group 1, one mouse died within 4 hours after injection of the prepared sample. Gros spathology indicated a severe irritation of the peritoneum. The other mice in this group displayed signs of malaise prior to cardiac puncture.

To determine whether the cause of death and the symptoms observed in group 1 were associated with the carrier in which the microorganisms of the sample were incorporated, the following tests were performed.

One half gram of the sample was incubated in 20 ml. of nutrient broth for 24 hours. A portion of the supernatant liquid was decanted into another tube of nutrient broth and incubated for an additional 48 hours. The resulting cell suspension was then centrifuged and washed twice with isotonic saline solution. This technique resulted in a suspension which was largely free of the carrier material and it contained about 3×10^8 organisms per ml. One milliliter of this suspension was injected intraperitoneally into five mice. No deaths or abnormal symptoms were observed.

One gram of the sample was incubated for 48 hours in 20 ml. of nutrient broth. After incubation, the material was autoclaved. The bacterial cell count was about 3×10^8 nonviable organisms per ml. One milliliter of this material was injected intraperitoneally into five mice. All of the five mice died within 16 hours, displaying symptoms of abdominal irritation and sensitivity.

One milliliter of the nutrient broth was injected intraperitoneally into five mice No deaths or abnormal symptoms were observed.

Although direct intraperitoneal injection of Thuricide into mice caused toxic symptoms, this effect was due to the carrier only. The organisms themselves had no toxic effect, nor was any virulence developed by serial passage through mice.

Persistence of *B. thuringiensis* in Blood of Mice Following Intraperitoneal Injection. Sixty white mice weighing 17 to 33 grams were assembled into six groups of 10 mice each. The animals were held in wire cages and fed a standard laboratory mouse diet and water ad libitum. Groups 1, 2, and 3 were injected intraperitoneally with 0.1 ml. of a 24-ml. nutrient broth culture of *B. thuringiensis*. Groups 4, 5, and 6 were injected with 0.1 ml. of a 24-hour broth culture of *B. cereus*, an organism which is generally considered as nonpathogenic under most conditions and is morphologically related to *B. thuringiensis*. This organism was included for comparative purposes.

Blood samples, 0.3 ml., were withdrawn by cardiac puncture from groups 1 and 4 after 24 hours, from groups 2 and 5 after 48 hours, and from groups 3 and 6 after 72 hours. Each blood sample was plated on tryptone glucose extract agar and the resulting *B. thuringiensis* or *B. cereus* colonies were counted.

The plate counts showed that both organisms persisted in the blood up to 48 hours. However, as neither organism was found at 72 hours, the persistence of *B. thuringiensis* was no greater than that of the strain of *B. cereus* used in this test.

Determination of Relative Pathogenicity of *B. thuringiensis* by Parenteral Administration into Guinea Pigs. The pathogenicity of *B. thuringiensis* for guinea pigs was compared with that of *B. cereus* and of *B. subtilis*, both considered to be nonpathogenic under most conditions. The method employed was that of Clark (*5*). The test organisms were cultured in glucose broth for 24 hours before injection. To obtain higher concentrations of organisms, 24-hour glucose agar slants were prepared and the organisms were washed off with 1 ml. of saline.

The groups of guinea pigs injected with the 24-hour broth culture received 4 ml. each. The groups injected with the slant washings received 1 ml. containing the growth from one slant. All injections were intraperitoneal. The animals were observed for 7 days after injection. Data are shown below.

Organism	Type of Culture	No. of Animals Injected	No. of Animals Surviving
B. thuringiensis	Broth	10	10
	Slant	10	3
B. cereus	Broth	5	5
	Slant	5	0
B. subtilis	Slant	5	5

Massive doses of the microorganisms are required to overcome the guinea pigs defense mechanism, because injection of the broth cultures caused no fatalities.

Inhalation Toxicity of *B. thuringiensis* Berliner in Mice. Inhalation toxicity was determined by placing 10 mice identified as Test Group 1, in an exposure chamber 30 × 30 × 30 cm. and dispersing Thuricide with a powder blower by means of compressed air. The animals were subjected to four exposures over a period of 6 days. The duration of each exposure was 15 minutes, during which time 10 grams of sample were dispersed. Between exposures the animals were housed in wire cages and were fed laboratory mouse diet and water ad libitum. The mice were weighed initially and at the end of the test. Observations were made of their reaction in the exposure chamber as well as of their general well-being throughout the test period.

In order to determine whether irritation to the lungs might result from the inhalation of only the carrier in which the active ingredient of the sample was incorporated, a portion of the test sample was sterilized by autoclaving and another group of 10 mice was subjected to the same exposure.

During repeated exposures of the mice to inhalation of the test material, no untoward reaction was observed in either group. Observations of their general well-being throughout the test period showed no departure from normal in either group, as was demonstrated also by normal weight gains for both groups. Gross pathology findings were negative.

The Allergenicity of Thuricide in Guinea Pigs. The procedure of Draize, Woodward, and Calvery (*6*) was employed for the determination of the allergenicity of Thuricide. Twenty white male guinea pigs were distributed into two groups of eight each and one group of four. The hair was removed from the back and flanks by close clipping. The sample was tested by the following methods.

Injection of a 0.1% Suspension in Water. Injections were made intracutaneously using a 25-gage needle. Ten sensitizing doses were administered, by injection, every other day for 3 weeks. Sites of injection were at random over the backs and flanks. The first injection was 0.05 ml., while the other nine contained 0.1 ml. each. Eight animals were used.

Topical Application on Abraded Skin. Ten sensitizing applications were administered every other day for 3 weeks. The abrasion for each application was made, at random, on the backs and flanks. The test material was applied with a powder blower, covered with an aluminum patch and taped in place. The first application was approximately 25 mg., while the other nine were approximately 50 mg. each. Eight animals were used.

Topical Application to Intact Skin. Ten applications were made in the same manner as on the abraded skin, except that the skin was left intact. Four animals were used.

Readings were taken 24 hours after the first application or injection to record any initial allergenic response as evidenced by the development of erythema and/or wheal formation. Two weeks after the tenth application or injection the challenge injection or application was made in the region of the lower flank, where no previous application had been made. The challenge dose was the same as that given in the first sensitizing dose. Twenty-four hours later readings were taken again for correlation with those obtained after the first injection or application.

Administration of Thuricide by injection or by application to abraded skin caused a slight erythema and edema, indicative of local irritation. There was no reaction from its application on intact skin. There was no evidence of any allergenic response by any route of administration.

Inhalation and Ingestion of Thuricide by Human Volunteers. Eighteen human subjects were employed in this experiment. All of the individuals were subjected to physical and laboratory examinations immediately before the start of the experiment. They then ingested 1 gram of the Thuricide in capsules daily for 5 days. In addition to oral ingestion, five of the subjects inhaled 100 mg. of the powder daily for 5 days. Inhalation was from an inhaler device (Abbott's inhalator) and both oral ingestion and nasal inhalation were used on alternate days. At the end of the 5-day test period, the subjects again received physical and laboratory examinations and again in 4 or 5 weeks later. In addition to these tests, the individuals who inhaled the insecticide also were subjected to x-ray examinations at the same intervals.

The physical examinations included a detailed history and records of height, weight, temperature, blood pressure, respiratory rate, pulse rate immediately after exercise and 30 and 60 seconds thereafter, and vital capacity (in the inhalation group). They also included evaluations of the genitourinary, the gastrointestinal, the cardiorespiratory, and the nervous sytems. Laboratory tests included routine urinalysis, with qualitative and quantitative (when indicated) urobilinogen determination, complete blood count, sedimentation rate, blood urea nitrogen, glucose, bilirubin, and thymol turbidity tests. All of the subjects remained well during the course

of the experiment. All laboratory findings were negative.

Determination of Hazard to Humans from Continued Random Exposure to Thuricide. Eight Bioferm employees in different parts of the manufacture and control of Thuricide production were observed during a 7-month exposure to:

WHOLE FERMENTATION BROTH. Exposures of 300 ml. to several thousand gallons per day.

MOIST BACTERIAL CAKE. Exposures of 50 grams to several thousand pounds per day.

EFFLUENT. Exposures from 300 ml. to several thousand gallons per day.

FINAL POWDER. It contained up to 15×10^9 viable spores per gram. Exposures of 10 grams to several thousand pounds per day. This material is ground, blended, and packaged. It is finely divided and dusts easily.

The formal record of the eight employees during exposure was free of complaints of any kind. Two of the employees who had been exposed to a greater extent than any of the others (total exposure 251 hours to all phases of production and control) were given comprehensive medical examinations. The two subjects were in excellent health and showed no evidence of chronic or acute damage of any kind from exposure to materials handled in the plant. The results of this record indicate that no hazard to health exists from prolonged and continued exposure to broths, moist cakes, or powders of Thuricide.

Acute Oral Toxicity of Thuricide. Thuricide was administered to rats in a 33% suspension in water containing 1% of carboxymethyl cellulose as a thickener. Administration was made by means of a syringe, having attached a hypodermic needle with a ball tip. The dose was placed directly into the animal's stomach. Doses up to 24 grams of Thuricide—estimated 2×10^{12} viable spores—per kilogram of body weight were administered to groups of 10 rats and the animals were observed for 1 week. No fatalities occurred nor were there any outward symptoms of toxicity. Gross and histological examination of tissues revealed no differences from the tissues of control animals.

Discussion

The tests which have been described have emphasized the harmlessness of the microbial insecticide Thuricide, and the active ingredient, *B. thuringiensis* Berliner, for warm-blooded, animals. The results of these tests partially satisfied the toxicity requirements of the Food and Drug Administration.

In addition, the area of lack of toxicity has been considerably extended in reports from other laboratories (*1*, *4*, *7–9*). This further work has included acute and chronic toxicity tests with chicks, laying hens, young swine and hogs, fish, adult honey bees, and honey bee larvae. In one of these tests (*4*), a group of New Hampshire Red laying hens received as part of their diet a daily supplement of 0.5 to 10 grams of Thuricide for 23 months. No significant differences in weight, appearance, or egg number and quality were observed between the test and control groups of laying hens.

One of the recurring questions about the harmlessness of *B. thuringiensis* to warm-blooded animals has been the important matter of the taxonomic grouping of this microorganism in the same genus with *Bacillus anthracis*. The morphological similarity of these two microorganisms is the basis for this classification. However, this does not by any means indicate that the characteristics of the two are interchangeable. The specific questions have been whether these two microorganisms can be confused with one another or mutate into virulent forms, and not whether *B. thuringiensis* has any of the virulent characteristics of *B. anthracis*. These questions have been answered, in the negative, in a full discussion by Steinhaus (*16*). In the manufacture of Thuricide during the last $2^1/_2$ years, no instance has been found of accidental contamination, or of mutation of *B. thuringiensis* into a virulent form for man, animals, or plants (*3*, *10*). In addition to the rigid quality control procedures applied (*3*), the final definitive test before the release of any batch of Thuricide is a mouse safety test described by Simmons and Gentzkow (*11*).

Finally, there have been no reports of toxicity of any kind to plants, animals, beneficial insects, or humans during the field application of nearly 1000 pounds of Thuricide in the 1957 and 1958 seasons.

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