BACTERIAL INSECTICIDES[1]

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I. Introduction

With the improvement in our ability to control insects has come the more specialized use of terms to describe the materials we use. Thus, the word insecticide has come to mean insect poison, and a microbial insecticide a poisonous substance produced by a microbe as opposed to disease caused by an entomogenous bacterium.

This becomes more complicated when one considers that in mammals, as well as in insects, bacteria usually penetrate the host by producing toxic substances. However, the distinction here is that the latter toxins are metabolic by-products of the growing and multiplying bacterium. Thus, to define our title, we wish to describe a type of bacterium that produces an endotoxin which is poisonous to many *Lepidoptera* and some other insects. It is analogous in insects to botulinus poisoning in humans. These bacteria are classified as varieties of two species, *Bacillus thuringiensis* var. *thuringiensis* Berliner and *Bacillus entomocidus* var. *entomocidus* Heimpel and Angus (52).

This type of insecticidal bacterium is not common, for most entomogenous bacteria must grow in the host before they overcome the natural resistance of the insect and kill it. An example of this is the disease of the Japanese beetle, *Popillia japonica* Newn., caused by the spore-former *Bacillus popilliae* Dutky. Spores of this bacterium must be ingested by beetle larvae and germination takes place in the gut. Multiplication of the bacteria at first occurs slowly in the gut, but penetration of the epithelium and entrance into the hemolymph (or blood) is accomplished rapidly. Additional, more rapid multiplication of the bacteria in the blood takes place until the insect is literally packed with sporulating, vegetative rods. The insect dies in two weeks or more after a protracted course of the disease. Obviously, there is no extremely toxic substance involved in killing the insect. This cannot be said, however, for the relatively rapid penetration of the gut. Essentially, *B. popilliae* is likely a pathogen by virtue of its ability to breach the gut wall and enter the body cavity. The speed and efficiency with which it accomplishes this might very well be due to a preformed, toxic material since growth is slow at this phase.

[1] Contribution no. 17, Insect Pathology Research Institute.

Bucher (21) described a sporeforming bacterium pathogenic for *Malacosoma pluviale* (Dyar) and *Malacosoma americanum* (Fab.). The bacterium[2] germinates and grows only in the gut, and requires a considerable time to kill the insect. There is no evidence of a highly poisonous, quick acting substance produced by the bacteria. Consequently, this organism is considered to cause a "true" disease. However, this is not to suggest that the bacterium does not produce substances harmful to the insect.

There are other examples of "true" insect diseases; however, sufficient has been reviewed to demonstrate the existence of two types of pathogenic bacteria: (a) bacteria that produce an insecticidal poison when grown outside the insect, and (b) bacteria that must grow in or invade the insect gut in order to kill. These bacteria may produce toxic materials while multiplying in the gut.

Oddly enough, the most effective insect pathogens are fastidious and are persuaded with difficulty to grow (*e.g.*, *B. popilliae*, *Bacillus alvei* Ches. and Chey., and Bucher's organism) on artificial media. It should be noted here that insufficient attention has been given to anaerobic methods for culturing these bacteria. It has been established for some time that the gut contents of many insects are strongly reducing at their normal pH (89).

It is one of the functions of insect pathology to provide information that will aid in the control of insect pests. We should then perhaps extend these introductory remarks one step further to consider the use of bacteria to control economically important insects. If a "true" disease pathogen is used, it may only be necessary to introduce the bacterium into the insect population at carefully selected points. The disease then spreads, by natural means, throughout large areas (*e.g.*, *B. popilliae*). If one is dealing with a microbial insecticide, then the bacterial poison must be applied so that it "blankets" the crop which is to be protected. This application must be applied as often as necessary to maintain an effective concentration on the crop.

The purpose of this paper is to describe a group of bacteria that are considered to be microbial insecticides. The toxin produced by the bacteria affects lepidopterous larvae and apparently some dipterous larvae. Over 80 species of susceptible *Lepidoptera* have been listed (99).

[2] Bucher has not yet named the organism but he has reported that it is extremely virulent; the LD_{50} for *Malacosoma pluviale* is in the vicinity of 100 spores per insect.

II. The Crystalliferous Bacteria

A. Early Investigations

In 1902, a sporeforming bacterium was isolated by Ishiwata from dying silkworm larvae. Ishiwata (according to Masera (71)) described his work with the bacterium in 1905 and he called the organism "Sotto-Bacillen".[3] Aoki and Chigasaki began working with the bacterium in 1911 and in a series of papers described the bacterium and the disease it caused when ingested by silkworm larvae (11,14). They noted that the bacterium was incapable of causing the disease unless old, sporulated cultures were fed to the insects. When this was done, the larvae became paralyzed within 60 to 80 min.

At approximately the same time, Berliner isolated a sporeforming bacterium from *Anagasta* (*Ephestia*) *kuehniella* Zell. which caused a disease in the larval stage of this insect (15). He described the bacterium in 1915 and named it *Bacillus thuringiensis* Berliner after the province Thuringia in which the work was done (16). Berliner's original isolate was lost, but Mattes reisolated the organism from *A. kuehniella* in 1927 and described the disease it caused in the flour moth (72). This strain (now known in Europe as "the German strain") was acquired by Porter in the United States who passed it on to Smith and to Steinhaus. The *B. thuringiensis* culture distributed to commercial and research laboratories in North America is believed to be Mattes' isolate.

Bacillus thuringiensis was used in field tests against the corn-borer, *Pyrausta nubilalis* (Hbn.), by European workers who reported excellent results over a period of four years (55,58,77,81). Mortality in sprayed plots was 96.8 to 99.2 per cent as compared to 81.7 to 87.5 per cent in unsprayed plots. That such exceptional results have not been obtained in more recent experiments (73) is likely due to the fact that in the former experiments the plants were sprayed and then colonized with insects, thus ensuring that the insects ingested the bacteria while burrowing into the stalk.

On the other hand, Husz (56) sprayed plants

[3] Sotto in Japanese signifies sudden collapse.

naturally infested with corn-borer and found that the number of entrance or bore-holes was reduced 50 per cent in the treated plots. Only 19 and 13 per cent of plants in two treated plots were infested, as compared to 32 and 27 per cent in the respective check plots.

The Metalinkovs, father and son, (82, 83) went on to test *B. thuringiensis* against several *Lepidoptera* in the field, including *Gelechia gossypiella* (Saund.), *Prodenia litura* F., *Sparganothis pilleriana* Schiff., *Clysia ambiguella* Hbn. and *Ephestia elutella* (Hbn.). The results reported were very encouraging (92).

B. Recent Investigations

In 1950, Jacobs tested a French product called "Sporeine," containing 10 per cent spores and 90 per cent bentonite, against *A. kuehniella* in a series of excellent experiments (59). He found that 0.1 to 0.3 per cent by weight of Sporeine mixed with flour protected against the flour moth. Higher concentrations (1.7 per cent) gave only partial control if the bacterial powder was merely dusted on the surface of the flour. Jacobs sent us the cultures of the bacteria he isolated from Sporeine and two of these are definitely *B. thuringiensis* var. *thuringiensis*.

At the same time (1949–1950) in North America, Steinhaus and Thompson were testing a polyhedrosis virus against the alfalfa caterpillar, *Colias philodice eurytheme* Bdvl. (93). Realizing that the virus disease was too slow in development to afford protection to the foliage before the infected insects died, Steinhaus began tests with bacteria, including *B. thuringiensis*. His conclusions were very optimistic and he reported that infected *C. eurytheme* ceased feeding within a few hours after ingestion of spores. The value of using *B. thuringiensis*, according to Steinhaus, was the rapid kill (within 48 hr), thus reducing the insect population below economic level, and preventing excessive loss of foliage (95).

To summarize, at the beginning of the last decade, the *B. thuringiensis* type of bacterium had been isolated, described, and tested successfully against a fairly wide variety of *Lepidoptera*. The fact that the old sporulated cultures were the virulent form of the bacterium had been noted by several workers (11, 55, 72, 95).

Toumanoff and Vago reported the isolation of a bacterium closely resembling *B. thuringiensis* and *Bacillus sotto* from silkworms dying of "flacherie" (110). Since the bacterium also resembled *Bacillus cereus*, these investigators named it *Bacillus cereus* var. *alesti* (after the region of Alés, in France). In a second paper they compared *B. sotto*, *B. thuringiensis*, and *B. cereus* var. *alesti* as to their cultural characteristics and came to the conclusion that these three bacteria were all varieties of *B. cereus* (111).

At the level of knowledge in 1952, this finding was justified. The colony and cellular morphology of *B. sotto*, *B. alesti*, and *B. thuringiensis* are superficially the same as *B. cereus*.

Essentially the characters that distinguish *B. cereus* from other species of *Bacillaceae* are as follows: *Bacillus cereus* is a gram-positive spore-former producing a vegetative rod 0.9 μ or more in width. It produces acetylmethylcarbinol, and does not ferment xylose or arabinose. A further criterion is the production of phospholipase C, since all *B. cereus* strains produce this enzyme (28, 29, 48, 76, 112). Angus (4), Toumanoff and Vago (110), and Smith *et al.* (91), prior to 1953, pointed out that both *B. sotto* and *B. cereus* var. *alesti* could be readily identified as *B. cereus* according to these tests.

Smith *et al.* noted that sporulated cultures of *B. thuringiensis* always had a large percentage of sporangia in which spores were slanted, that is, were canted to one side in relation to the main axis of the bacillus (91). Since this appeared to be a fairly constant characteristic of this insect pathogen, they tentatively recommended that the name *B. thuringiensis* be unchanged on practical grounds, although academically the bacterium resembled *B. cereus* (according to Steinhaus and Jerrel (97)).

At this time, two important discoveries were made which eventually led to an understanding of the mode of action of these bacteria. First, Hannay in London, Canada, noted the presence of a second body along with the spore in each sporangium of *B. thuringiensis*,[4] and in 1953 he published a paper with excellent photographs showing this body, which he described as a "diamond-shaped crystal" (42). By using Robinow's technique of an air-mounted nigrosin smear of sporulating cultures, he was able to

[4] Both Berliner (16) and Mattes (72) had reported the presence of the parasporal body in the sporangium, but neither had connected this observation with the pathogenicity of the bacterium.

define the crystal and spores very clearly. More than this, he made an astute speculation "as to the possible association of inclusions with pathogenicity." Electron micrographs of the crystal and the spore (figure 1) and another of the crystal alone (figure 2), were prepared by R. E. Monro and R. W. Horne (84).

Second, Angus (2) in Sault Ste. Marie, Canada, working with *B. sotto*, had repeated and confirmed Aoki and Chigasaki's original observa-

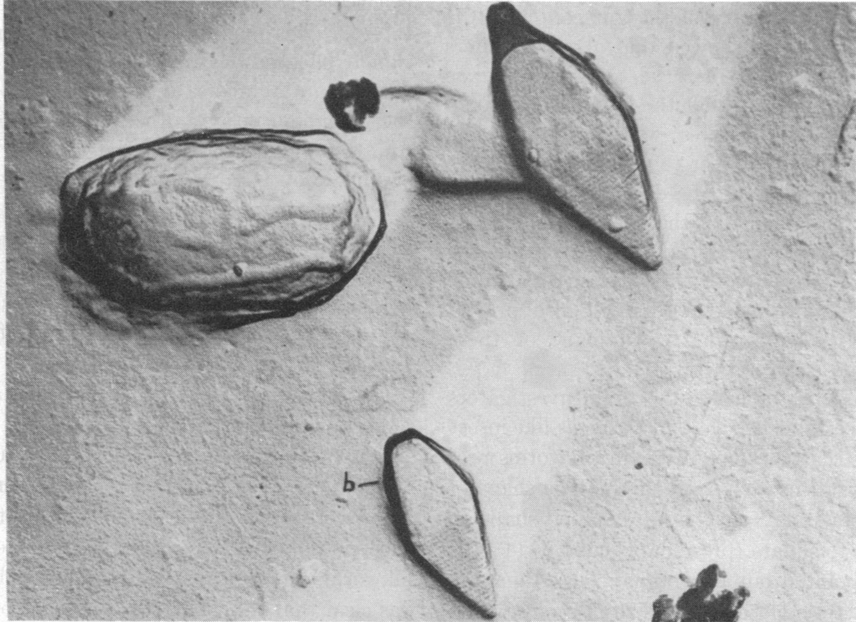

Figure 1 (*top*). Spore and crystals of *Bacillus thuringiensis* var. *thuringiensis* Berliner. (From Monro (84)).

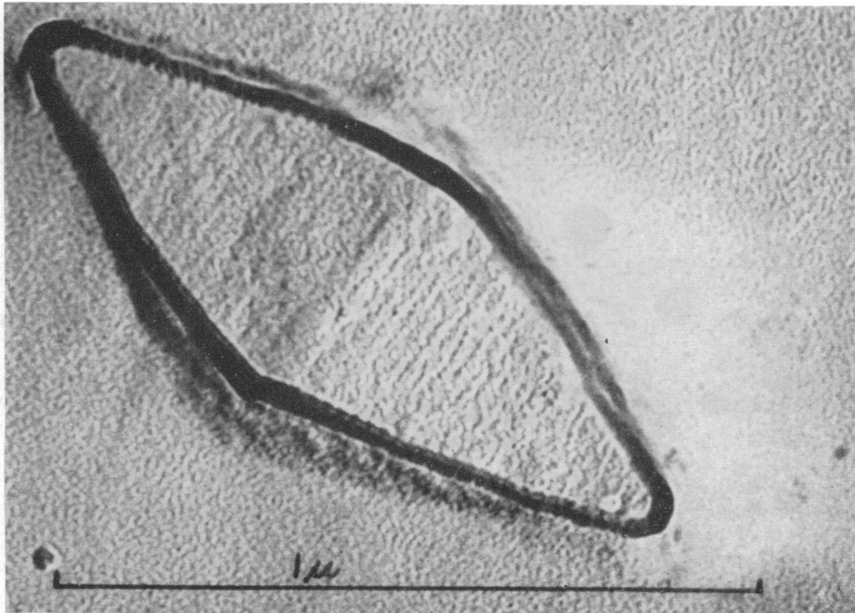

Figure 2 (*bottom*). A crystal of *B. thuringiensis* var. *thuringiensis*. (From Monro (84)).

tions of the toxicity of sporulated cultures of *B. sotto* for the silkworm. He demonstrated that gut contents from silkworm larvae, mixed with sporulated cultures and filtered free of bacteria, extracted a "toxic substance" that paralyzed silkworms in 60 to 80 min. when fed. Angus was aware of the alkalinity of the gut contents (pH 9.5 to 10.4) and he showed that alkaline buffers could be used to extract the toxin. There was one anomaly here, in that the best yield of toxin from sporulated cultures was obtained using buffers with a pH higher than was found in the silkworm gut. This will be discussed later. Since Hannay pointed out that the crystals were alkali-soluble, Angus separated the crystals from the spores and extracted the former with alkaline buffers. The filtrates he obtained also proved toxic for the silkworm. A summary of these experiments is given in table 1.

Subsequent studies by several investigators have since established that all *B. cereus*-like insect pathogens that cause paralysis in silkworms produce a crystal in each sporangium. The shape of these crystals may vary from diamond-shape to triangular to square (presumably cuboid) (43, 97, 115). As pointed out by Hannay (43), there are several bacteria closely related to *B. cereus* which produce parasporal bodies, *e.g., Bacillus laterosporus, Bacillus medusa, Bacillus finitimus* (43, 51).

C. Taxonomy

Since the discovery of the crystal in *B. thuringiensis* and in *B. sotto*, insect pathologists have looked for this characteristic in bacteria found in insects dying from this type of disease. Several isolations have been made and these are listed in table 2. This made available a number of very similar bacteria described by a variety of names and culture numbers, and for practical purposes the taxonomic situation became rather confused. An attempt to bring some order to the situation was made by Delaporte and Béguin in 1955 (31). They examined several strains of cystalliferous bacteria including Vago's "souche anduze." Their conclusions were that *Bacillus sotto, Bacillus cereus* var. *alesti, Bacillus thuringiensis* (French strain), and "anduze" were all strains of *Bacillus thuringiensis* Berliner.

Heimpel and Angus collected the available crystalliferous pathogens and examined them as a group (52). Among these were two cultures from Steinhaus (91, 94). Neither of the latter isolates produced acetylmethylcarbinol nor did they produce phospholipase C. Both were toxic for the silkworm, but to a markedly different degree. It was clear that these bacteria could not be considered varieties of *B. cereus* despite the fact that otherwise they resembled this species. The only alternative was to create a new species, namely,

TABLE 1*

Effect of feeding and injecting larvae of Bombyx mori L. with fractions of an alkali-treated culture of Bacillus sotto

Culture	Method of Dosing Larvae	
	By feeding	By injection
Original culture		
Spores and crystals (1×10^5 spores per larva)	Paralysis within 4 hr, septicaemia within 12 hr	Septicaemia within 12 hr, no paralysis
Alkali-treated culture		
1. Spore fraction (1×10^7 spores per larva)	No effect	Septicaemia within 12 hr
2. Supernatant	Paralysis within 4 hr, no septicaemia	No effect
3. Supernatant dialyzed	Paralysis within 4 hr, no septicaemia	No effect
4. Supernatant heated at 70 C for 30 min	No effect	No effect

* After Angus (2).

TABLE 2
Crystalliferous bacteria

Name	Host	Authority	Taxonomic Re-assignment	Authority
Bacillus sotto (Ishiwata)	*Bombyx mori* L.	(11)	*Bacillus thuringiensis* var. *sotto* Aoki and Chigasaki	(52)
Bacillus thuringiensis Berliner	*Anagasta* (Ephestia) *kuehniella* (Zell.)	(15, 72)	*Bacillus thuringiensis* var. *thuringiensis* Berliner	(52)
Bacillus sp.	*Aphomia gularis* Zell.	(94)	*Bacillus entomocidus* var. *entomocidus* H. and A.	(52)
Bacillus sp.	*Plodia interpunctella* Hbn.	(94)	*Bacillus entomocidus* var. *subtoxicus* H. and A.	(52)
Bacillus cereus var. *alesti* Toumanoff and Vago	*Bombyx mori* L.	(110)	*Bacillus thuringiensis* var. *alesti* Toumanoff and Vago	(52)
Bacillus cereus var. *alesti* "anduze" strain	Silkworm rearing litter	By Vago (31)	*Bacillus thuringiensis* var. *alesti* Toumanoff and Vago	(52)
Bacillus sp.	*Heliothis obsoleta* F.	(67, 68)	*Bacillus thuringiensis* var. *thuringiensis* Berliner	(52)
Bacillus dendrolimus	Talalaev *Dendrolimus sibericus* Tshtv.	(105)	*Bacillus cereus* var. *dendrolimi* Talalaev	(117)
Bacillus thuringiensis Berliner	*Plodia interpunctella* Hbn.	By Weiser (119)	*Bacillus thuringiensis* var. *thuringiensis* Berliner	(52)
Bacillus sp.	*Galleria mellonella* L.	(90, 117)	*Bacillus cereus* var. *galleriae* Toumanoff and Vago	(117)
Bacillus thuringiensis Berliner	*Galleria mellonella* L.	(60)		

Bacillus entomocidus and its variety *B. entomocidus* var. *subtoxicus*.

In agreement with Smith *et al.*, and Delaporte and Béguin, the view was taken that the presence of the crystal was a stable characteristic that allowed the separation of an insect pathogen from similar but nonpathogenic *B. cereus* strains, and that this served a practical purpose for bacteriologists. Consequently, the retention of *B. thuringiensis* as a valid species was recommended and a key for the species belonging to the "*B. cereus* group" was proposed. This key, which is reproduced here, proposed *sotto*, *alesti*, *anduze*, and *thuringiensis* as varieties of the same species.

KEY TO THE SPECIES OF THE "*Bacillus cereus* GROUP"

Mesophilic (good growth between 28 and 35 C), aerobic (usually facultative anaerobes). Spores ellipsoidal to cylindrical, paracentral to subterminal, walls thin. Sporangia not distinctly bulged. Gram-positive.

A. No parasporal body present
- (a) Acid from xylose and arabinose with ammoniacal nitrogen. Acetylmethylcarbinol not produced. Phospholipase C not produced.
  - 1. *Bacillus megaterium*
- (aa) No acid from xylose and arabinose. Acetylmethylcarbinol produced. Phospholipase C produced.
  - (b) Saprophytic, sometimes pathogenic but not causing anthrax; sometimes motile.
    - (c) Growth on agar not rhizoid.
      - 2. *Bacillus cereus*
    - (cc) Growth on agar rhizoid; seldom motile.
      - 3. *Bacillus cereus* var. *mycoides*
  - (bb) Pathogenic. Causative agent of anthrax; nonmotile.
    - 4. *Bacillus anthracis*

B. Parasporal bodies present
(a) Parasporal body released from sporangium and separated from the spore in two to six days; pathogenic for *Lepidoptera* larvae.
(b) Acetylmethylcarbinol produced. Phospholipase C produced.
(c) Pellicle formed in nutrient broth. Pellicle breaks into flakes in shaken culture. Low toxicity for the silkworm.
5. *Bacillus thuringiensis* var. *thuringiensis*
(cc) No pellicle formed in nutrient broth, body of broth evenly turbid, shaken culture gives even dispersal. Highly toxic for *Lepidoptera*.
(d) No pigment formed when grown on egg-yolk agar.
6. *Bacillus thuringiensis* var. *sotto*
(dd) Rosy pigment formed in agar after several days growth on egg-yolk agar.
7. *Bacillus thuringiensis* var. *alesti*
(bb) No acetylmethylcarbinol produced. No phospholipase C produced.
(c) Acid from trehalose, levulose, and glucose after 20 days incubation at 32°C. Highly toxic for many *Lepidoptera*.
8. *Bacillus entomocidus* var. *entomocidus*
(cc) No acid from trehalose, levulose, and glucose after 20 days. Low toxicity for certain *Lepidoptera*.
9. *Bacillus entomocidus* var. *subtoxicus*
(aa) Parasporal body firmly attached to spore, even after months of storage. Acid from cellobiose after 48 hours incubation. Nonpathogenic for certain *Lepidoptera*.
10. *Bacillus finitimus*

Careful study of the literature established that priority should be given to *Bacillus thuringiensis* var. *thuringiensis* Berliner as the type species.

Recently Toumanoff and LeCoroller published an alternative scheme for the taxonomy of the "*B. cereus* group" (117). These investigators do not think that the presence of a toxic crystal in a bacterium is sufficient to justify species status. They proposed a key which divides *B. cereus* into two sections, the "acrystallophorous" and the "crystallophorus" groups. The crystallophorous group is then broken down into varieties of *B. cereus* on the basis of the action on Loeffler's medium, coagulated serum, and egg-yolk agar. In some instances, the variety differentiation is based on the rate of action on the media. Further differentiation is made on the basis of the host from which the bacterium was isolated; indeed, heavy emphasis is placed on the host since the authors state, "The biology of the host, its alimentary regimen and its environment are the factors which certainly govern the pathogenic effect and also the biochemical and morphological characteristics of the pathogenic germ".[5]

These writers propose that the varieties found in various insects arose as a result of passage from one species of insect to another in the same environment and that the effect of the host on the bacterium is to change it, implying for example that *B. thuringiensis* might pass from *A. kuehniella* to *Plodia interpunctella.* Hbn. and in the latter host it becomes altered thus providing a new variety of the bacterium. This is an interesting and revealing speculation; however, a great deal of careful experimentation is required before such a theory is acceptable as a basis for taxonomic differentiation of varieties of bacteria.

Essentially then the difference of opinion between these teams of researchers is whether the isolates of crystalliferous bacteria should be made varieties of *B. cereus* or be made varieties of the valid species, *B. thuringiensis*. Heimpel and Angus take the view that there is justification for retaining *B. thuringiensis* var. *thuringiensis* as type species of crystalliferous bacteria since for practical purposes it is less restrictive taxonomically (52). Most bacteriologists would agree that bacteriological nomenclature is but a system for the practical differentiation of strains of bacteria. Anyone who takes an academic stand, in defence of the species concept, is on uncertain ground.

To distinguish the crystalliferous bacteria from *B. cereus* one must be sure that the crystal is a stable characteristic. Heimpel and Angus stated that these bacteria, grown on nutrient agar

[5] "La biologie de l'hôte, son régimen alimentaire et son milieu environment sont facteurs qui régissent certainement l'effet pathogène et aussi les caracteres biochimiques et morphologiques du germe pathogene."

(Difco), at 30 to 33 C, invariably produce crystals (52). However, Vaňková reported that certain strains grown on meat-peptone agar at "room temperature" for 6 months lost the ability to form crystals (119). Although her medium very probably varies from that used in North America, it is possible that these cultures were overgrown by a *B. cereus* contaminant. Unpublished work by Heimpel indicates that in experimental culturings of mixed inocula of *B. thuringiensis* varieties and *B. cereus*, the latter species will outgrow the crystal-former in one to several months. This has serious implications for an industrial firm producing the crystal-former.

D. Stability of Crystal Production

Toumanoff *et al.* stated that following prolonged passage in broth culture or prolonged growth on nutrient agar at pH 9.0 to 9.5, the loss of crystals in *B. thuringiensis* var. *alesti* was noted (115). In the former treatment the strain remained asporogenic and acrystalliferous after return to nutrient agar at neutral pH. However, when asporogenous bacteria from the high pH treatment were injected into *Galleria mellonella*, the ability to sporulate and form crystals returned. These strains were toxic for silkworms, but less so than the original crystal-forming *B. thuringiensis* var. *alesti* strain. This brings up the question as to whether Toumanoff was dealing with *B. thuringiensis* var. *alesti* throughout these tests or whether he inadvertently got a *B. thuringiensis* var. *thuringiensis* contaminant during his *Galleria* injection experiments; in this connection Fitz-James and Young (35) examined a large series of crystal-forming and non-crystal-forming sporeformers and found that *B. thuringiensis* var. *thuringiensis* spores have 10 times more residue phosphorus (after RNA-P and DNA-P are removed) than do var. *alesti*, var. *sotto*, or *B. cereus*. Again they found that *B. thuringiensis* var. *alesti* spores have twice as much DNA-P as *B. cereus*, *B. thuringiensis* var. *sotto* and *B. thuringiensis* var. *thuringiensis*. Fitz-James and Young also listed a strain from Toumanoff (B-1) which they stated was "isolated from larvae of *Galleria mellonella* into which an asporogenous culture of a *Bacillus* sp. had previously been injected." This was undoubtedly one of Toumanoff's asporogenous cultures of *B. thuringiensis* var. *alesti* (115). However, Fitz-James' results show that this B-1 strain had 10 times as much residue phosphorus as did *B. thuringiensis* var. *alesti* and *B. cereus* strains, and almost exactly the same amount of DNA-P as *B. cereus*. In other words, Toumanoff's treatment converted a strain of var. *alesti* to var. *thuringiensis*, and this conversion of var. *alesti* was so complete that the B-1 strain lost its ability to form the characteristic red, var. *alesti* pigment (115), and was reduced in virulence for the silkworm (a characteristic of var. *thuringiensis* previously reported (52)).

On the other hand, Fitz-James and Young (35) stated that treatment of *B. sotto* with a "mutagenic agent" caused the irreversible loss of ability to form crystals and his later tests showed that the treated acrystalliferous strain indeed resembles *B. sotto* as to phosphorus content. This then is a proved conversion of a crystalliferous bacterium to an acrystalliferous form. Another case of the reverse reaction was reported by Toumanoff (116) when he injected several cultures of *B. cereus* into the wax moth. One strain, A-30, he claims, became crystalliferous and Fitz-James showed that this strain resembled *B. thuringiensis* var. *thuringiensis* in phosphorus content of the spores. Fitz-James and Young also showed that two crystals, one triangular and one small and diamond-shaped, are formed in each sporangium (35).

LeCoroller also described several techniques whereby the ability to produce crystals could be manipulated (64).

Most investigators agree that under normal conditions of temperature and pH on nutrient agar (with a composition resembling that of Difco), the production of crystals by pure cultures of crystalliferous bacteria is a surprisingly constant character.

III. The Formation and Characterization of the Toxic Crystal

A. Development

Rigorous treatment of the bacteria (such as Fitz-James' formalin treatment (35)) can cause the loss of ability to form a parasporal body, which suggests that crystal production may be under genetic control. This brings up the interesting question as to why the crystal is formed. Subcultures of *B. thuringiensis* var. *sotto*, now 45 years old, are in our possession and upon incubation on nutrient agar at 33 C they invariably produce crystals. Certainly such extended saprophytic growth, without contacting an insect,

should have a degenerative effect on the ability to form crystals if this ability has selective value in the insect. If var. *sotto*, a silkworm pathogen, is injected into the larval silkworm hemocoele, very little sporulation takes place, and hardly any crystals are produced. *B. thuringiensis* var. *sotto* actually produces more toxin on artificial, solid media than it does in this insect host. In broth culture most crystalliferous bacteria require aeration to sporulate and form crystals. Since the crystal is protein without phosphorus content (6, 43), it is unlikely that it is an aborted spore. In one interesting theory the crystal has been compared to a garbage pail with a locked lid (a very apt if unsavoury analogy). If an undesirable substance is produced as a metabolic by-product of sporulation, it might be removed from the soluble environment by crystallization. This might be the explanation for the relatively great chemical inertness of the crystal. Certainly this is a field of investigation well worth considerable effort.

Young and Fitz-James (121) have shown that the elongation of the two chromatin bodies, in a vegetative rod, into a filamentous structure along the axis serves as reliable cytological evidence of the changeover from vegetative rod to sporulating cell. At this point growth and nucleic acid synthesis cease. At any time before this stage is reached, the addition of 8-azaguanine inhibits sporulation and, as a result, crystal formation. The addition of the analogue after this stage does not inhibit crystal formation.

Again, Young and Fitz-James (120) point out that the parasporal crystal appears only after the vegetative cell is committed to sporulation and Monro confirms this by pointing out "that crystal antigen is absent in vegetative cells but arises during sporulation" (84). They suggest that the crystal is synthesized from small molecular weight compounds into a final form of protein which at first, in the early stages of crystal formation, is soluble at a lower pH than is the "mature" crystal, which dissolves at pH 11.5. They propose that the formation of S—S bonds between the protein molecules accounts for this increase in insolubility with age, since the "mature" crystals become soluble at a lower pH upon addition of thioglycolic acid.

Hannay stated that crystal formation commences in cells containing either two or four chromatic bodies (43). When cells containing two chromatic bodies sporulate, the crystal does not appear until the spore reaches a considerable size. In sporulating cells with four bodies, spore and crystal formation is synchronized. The crystals are usually *octahedra* with a *tetragonal* face. According to Hannay, the crystals are plastic, and may be warped and distorted easily by manipulating the beam intensity of the electron microscope. Steinhaus and Jerrel (97) and Toumanoff and LeCoroller (117) have reported other shapes of crystals varying from triangular to cuboidal. Monro worked with a strain of *B. thuringiensis* var. *thuringiensis* that produced diamond-shaped crystals that, when viewed from the end, were square in appearance (84). Both Steinhaus and Jerrel (97) and Hannay (43) have reported cells containing two crystals. Hannay (43) reported that the crystal had a regularly serrated surface but was unable to tell whether these serrations were the outer edges of plates stacked one on top of the other, or represented spirally wound chains of molecular groups. He reported (43) that the crystal is not surrounded by a membrane as had previously been postulated (44). Hannay stated the crystals stain readily with most biological dyes, particularly the acid stains. However, the general staining reactions suggested a small number of free reactive groups in the intact crystal.

B. Chemistry

Although only three studies of the chemical composition of the crystal have been accomplished, we now know the crystal is a protein (6, 44, 84). Hannay and Fitz-James stated that *B. thuringiensis* var. *thuringiensis* crystals have 17 per cent nitrogen and a slight amount of phosphorus but later Hannay (43) reported that the crystal has no phosphorus. Angus showed *B. thuringiensis* var. *sotto* crystals are similar, containing 17.5 per cent nitrogen and no phosphorus. It has been suggested (6, 43, 44) that the crystal is a homogeneous substance but more proof of this point is required; however, if substances other than the toxic protein exist in the crystal, they are present in very small quantities.

To study the protein of the crystal, several ingenious methods have been devised for recovering the crystals free from spore contamination. Hannay and Fitz-James used two methods based on the tendency[6] of the *B. thuringiensis* var.

[6] *Bacillus thuringiensis* var. *sotto* is the best strain from which to obtain crystals with this method since its spores are less stable than those from *B. thuringiensis* var. *thuringiensis*.

thuringiensis spore to germinate in distilled water, leaving "ghost" spores and crystals, separable by differential centrifugation; the other method involved the mechanical destruction of the spores in a Mickle vibrator followed by differential centrifugation (44). Angus, working with *B. thuringiensis* var. *sotto*, used Hannay's former method and also worked with toxin dissolved in 0.05 N NaOH and the protein reprecipitated at pH 4.4 by the addition of acetate buffer (pH 4.1, 1 M) (6). This last step is very critical and any variance from the described procedure may cause biological inactivation of the toxin (witness Vaňková's report (119)).

Finally, Angus devised a method based on germination of the spore, followed by treatment of the suspension with a fluorocarbon (10). This heavy fluid sweeps the spore "ghosts" from the water suspension, leaving the crystals in the aqueous phase. This is, perhaps, the quickest and most thorough method for obtaining spore-free crystal preparations.

Preliminary chemical studies on the analysis of acid hydrolyzates of crystals have been reported (44). The results of such an analysis of *B. thuringiensis* var. *sotto* crystals are presented in table 3 (6). The amino acids present are not unusual in variety or in quantity compared to similar studies on other bacterial species.

Considerable work has been done on the solubility of the crystal. Hannay and Fitz-James (44) showed that *B. thuringiensis* var. *thuringiensis* crystals did not go into solution until pH 11.8 was reached. Fitz-James *et al.* also demonstrated that solution of var. *alesti* crystals was accomplished at pH 11.0 to 12.2 (34). A summary of their findings is reproduced in table 4.

TABLE 3*

Amino acid composition of Bacillus sotto toxin and crystalline inclusions†

Amino Acid	Crystalline Inclusions	Toxin	
		Average	Range
Arginine	9.4	9.6	9.5–9.7
Lysine	4.2	3.6	3.5–3.9
Cysteine and/or cystine	1.1	1.2	1.2–1.3
Histidine	1.7	2.7	2.7–2.8
Aspartic acid	9.5	9.6	9.3–10.2
Glutamic acid	12.9	11.8	11.6–12.0
Glycine	2.7	3.2	3.1–3.3
Serine	5.6	4.8	4.7–4.9
Alanine	3.2	2.8	2.7–2.9
Proline	6.7	7.5	7.4–7.6
Tyrosine	3.9	6.8	6.6–7.0
Threonine	5.2	4.5	4.3–4.7
Methionine	0.6	1.3	1.3–1.4
Phenylalanine	7.4	8.6	8.5–8.7
Valine	5.0	5.3	5.2–5.4
Leucine and/or isoleucine	10.4	11.2	11.2–11.3
Tryptophan‡	2.1	2.6	2.5–2.7
Total	91.6	97.1	

* After Angus (6).

† Estimated by paper chromatography of acid hydrolyzates, and expressed as grams of amino acid residues per 100 g of protein analyzed.

‡ Tryptophan was determined separately.

C. The Mode of Action

1. The host. The crystalliferous bacteria have never been isolated from any other source than insects, and we must therefore leave the test tube and study the behavior of the pathogen in what appears to be its "natural" environment. At the risk of being tedious, we would like to digress at this point to describe the insect from the physiological and pathological viewpoint. The larva appears to be the only stage of the insect susceptible to infection by these bacteria, and the following description refers to this stage, and particularly to larvae of *Lepidoptera*.

The insect larva is essentially a digestive tube with a body wall around it. It has an open blood cavity filled with a fluid that has many functions (including some attributed to the liver in vertebrates) and carries out most functions of the vertebrate blood. Various types of blood cells are present, some of them capable of phagocytosis, but not nearly as numerous as in vertebrate blood. The pH of insect blood is usually slightly acid (5.6 to 7.0) and it is at its lowest buffering capacity at normal pH. The heart is a tubular organ located dorsally, which by rhythmic contractions draws the blood in through lateral openings and pumps it forward to bathe the brain whence it flows slowly back ventrally to the posterior end through the hemocoele.

The digestive tube is uncomplicated, consisting first of a crop or foregut lined with a coating of the exoskeletal material which forms the outer body wall. This lining is impermeable to water. The foregut pH is usually that of the food, and in leaf feeders is generally slightly acid. The

TABLE 4*

Effect of feeding various fractions of Bacillus cereus var. alesti to silkworm larvae

Fraction No.	Source and Method of Preparation	Description of Preparation	Conclusion of Feeding Tests
A_1	Crystals completely separated from disrupted spores	Water suspension	Highly toxic
A_2	Crystals completely separated from whole spores mechanically	Water suspension	Highly toxic
A_3-3	pH 10.5 wash of partially purified crystals	A liquid (no precipitate at pH 4.5)	Slightly toxic (15% of larvae paralyzed in 24 hr)
A_3-4	Crystals, previously washed at pH 10.5, extracted at pH 11.8 to 12.0 = alkali extract of crystals, adjusted to pH 5.0 and centrifuged	Supernatant (freeze-dried)	No detectable toxicity
A_3-5		Residue: reprecipitated and washed (freeze-dried)	Toxic
A_3-6	Crystal gel remaining after alkali dispersion, water-washed	Wet sample	Highly toxic
A_3-7		Dry sample	Highly toxic
A_3-8	Spores repeatedly washed in alkali and acid	Water suspension	No immediate paralysis (20% dead in 16 hr, 50% dead in 50 hr)
A_3-9	A_3-8 spores completely disrupted	Freeze-dried powder	No effect on larvae
A_4-1	Growing vegetative cell protein extracted at pH 11 to 12	Freeze-dried at pH 4.5	Nontoxic
A_4-2	Sporulating cell protein extracted at pH 10.5	Freeze-dried at pH 4.5	Toxic

* After Fitz-James *et al.* (34).

foregut is separated from the midgut by a strong, valvelike sphincter. The flaps of the valve in most species contain cells which continuously secrete a membrane and this thin tubular structure (the peritrophic membrane) apparently protects the delicate midgut cells from damage by sharp particles of food. The midgut secretes digestive enzymes and buffering compounds, and absorbs the products of digestion. Anatomically, the midgut may be distinguishable into two or more regions on the basis of cell types, and usually the pH of the contents in each anatomical region is different. The gut contents are highly buffered and in *Lepidoptera* are as a rule alkaline. Some species have a low pH (7.0 to 9.0) of the midgut contents while in others it is relatively high (9.0 to 10.4). The midgut in many *Lepidoptera* is strongly reducing in action. Behind the midgut is the hindgut with its connected Malpighian tubules which serve as kidneys, and the heavily muscled rectum and anus. The hindgut and rectum are also lined with invaginated exoskeletal material. The pH of the hindgut and rectum is usually slightly acid to slightly alkaline (47, 50, 51).

The larval stage is a precarious one in the insect's life. *Lepidoptera* larvae must have continuous access to food if they are to remain healthy and complete development. Many feed continuously (*e.g.*, the silkworm); other species are meal feeders (*e.g.*, the tent caterpillars) and feed four or five times a day, resting between meals. When larvae are feeding, buffers are secreted at a maximal rate and the gut contents are at their highest pH. If the insect stops feeding, the pH in the gut falls steadily and apparently buffer secretion slows. The pH returns to strongly alkaline only food is reintroduced into the gut.

2. Host-pathogen relationships. Insect pathologists generally agree that many of the *Lepidoptera*, such as the silkworm, are resistant to saprophytic bacteria, including *B. cereus*, because of the high pH in the midgut (48, 51, 66, 92). Insects with a pH in the midgut that lies within the

range of good bacterial growth (pH 6.0 to 8.5) (*e.g.*, the larch sawfly and the codling moth) are susceptible to certain bacteria such as *B. cereus* (45, 46, 48, 104) and *Serratia marcescens* (49, 103). Some insects with a similar pH of the midgut contents are, however, resistant to *B. cereus* (*e.g.*, *A. kuehniella*); indeed in *Anagasta*, *B. cereus* spores germinate and then after a period the rods show granules, disintegrate, and disappear (52). Obviously then, there are other mechanisms of resisting bacterial infection acting in the midgut besides low hydrogen-ion concentrations.

3. General paralysis. Aoki and Chigasaki had pointed out that silkworms became paralyzed after feeding on sporulated cultures of *B. thuringiensis* var. *sotto* (11). Angus (1, 2) took this a step further, demonstrating that crystals separated from such preparations also produced general paralysis in silkworms. He later showed that other strains and species of crystalliferous bacteria produced the same symptoms in silkworms; and that the hornworms, *Protoparce quinquemaculata* (Haw.) and *Protoparce sexta* (Johan.) (4, 87), and the Chinese oak silkworm, *Antheraea pernyi* Guérin, also exhibited a general paralysis when fed the organism (53).

This general paralysis, at first thought to be symptomatic of the toxic effect in insects, soon proved to be an anomaly. Studies on the effect of *B. thuringiensis* var. *thuringiensis* on the alfalfa caterpillar *C. eurytheme* by Steinhaus (95) showed that the insects stopped feeding shortly after ingesting sporulated cultures, and died within 24 to 48 hr without exhibiting general paralysis. As other *Lepidoptera* were tested, it soon became obvious that general paralysis was the exception rather than the rule, but one symptom was common throughout the species tested; the insects invariably stopped feeding within a few minutes after ingesting the toxin. Obviously, the toxin was affecting a few species in a fashion distinctly different from its action on the majority of *Lepidoptera* larvae.

4. Gut paralysis. The foregoing point was finally resolved when it was shown that all susceptible *Lepidoptera* suffer from a gut paralysis shortly after feeding on sporulated cultures or on crystals. In a series of experiments involving the use of X-ray photography to trace the movement of barium sulfate, Heimpel and Angus demonstrated that food ingested with toxin ceases to move through the gut (53). It was obvious that the gut in the infected larvae had ceased to function. These studies confirmed observations by Vaňková who, while dissecting infected *Euproctis phaeorrhoea* larvae, noted that "intestinal function" ceased a few hours after feeding (119). The cause of this paralysis of the gut has not yet been elucidated; however, there can be little doubt that it is a quite different effect of the toxin than that in the silkworm and other insects which exhibit a general paralysis 1 to 7 hr after gut paralysis occurs. In the latter, the toxin is ingested and reaches the midgut in 3 to 5 min. Five minutes later the blood pH begins to change from the normal pH 6.8 (fifth instar larvae) and becomes more alkaline (8, 9). The increase in blood pH parallels the appearance of the general paralysis (see figure 3), and is accompanied by a concurrent decrease in pH of the highly alkaline gut contents. The obvious explanation is that the toxin acts very rapidly on the epithelium of the midgut, sufficiently destroying its integrity to allow the highly buffered alkaline gut contents to leak into the relatively poorly buffered blood. Injections of nontoxic buffer sufficient to raise the pH of the blood of normal feeding insects to pH 8.0 brought on a general paralysis indistinguishable from that induced by the toxin. These experiments were repeated with the hornworms and the Chinese oak silkworm with precisely the same results. In a representative group of

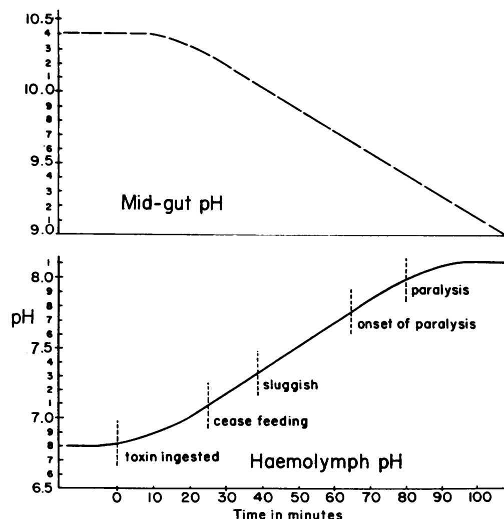

Figure 3. Changes in pH of the gut contents and blood of *Bombyx mori* larvae after ingesting crystals from *Bacillus thuringiensis* var. *sotto*.

susceptible insects that do not exhibit general paralysis, there is no increase in blood pH (53). Since sterile buffer injections in the latter brought about a general paralysis identical to that seen in infected silkworm, the following conclusions were reached:

General paralysis occurs in a limited group of *Lepidoptera* larvae having a high pH in the gut, referred to as a type I host and reaction. This paralysis is caused by an alkaline blood-pH change to the extent of 1.0 to 1.5 pH units; this change in pH is caused by the action on the epithelium of the midgut of the toxin produced by crystalliferous bacteria. Type I insects also suffer a gut paralysis which becomes evident only if sublethal doses of the toxin are fed, in which case the gut paralysis is followed by a drop in gut pH, allowing the spores to germinate and septicemia to develop.

The type II insect (represented by the bulk of *Lepidoptera* species tested) is afflicted by gut paralysis a few minutes after ingesting the toxin, and ceases feeding. There is no increase in blood pH and consequently no general paralysis; the pH of the gut falls slowly as it does in a starved insect (47), and this allows good germination and multiplication of bacteria. Since these insects die in a shorter time than that required to kill by inanition, there is little doubt that the growing bacteria hasten their demise.

This might be the explanation for the anomaly noted and commented on by Vaňková (119). Vaňková used two type II insects in her experiments, namely, *Lymantria dispar* L. and *E. phaeorrhoea*. She fed a 7-day-old culture of *B. thuringiensis* (Czech strain 058) to *E. phaeorrhoea* larvae and obtained a mortality of 100 per cent in 6 days. She found that 90 per cent of larvae fed vegetative rods died in 8 days (this is most unusual and will be discussed later). Spores (with the crystals removed by treatment with 0.1 N NaOH) of this strain fed to *E. phaeorrhoea* caused 68 per cent mortality in 7 days. Vaňková then repeated these experiments with a culture she claims is an acrystalliferous strain of *B. thuringiensis* (Czech 058), which is listed in the Czech culture collection as *B. thuringiensis* (acrystalliferous-059). She found that neither the vegetative cells nor the spores of the acrystalliferous strain alone caused death of *E. phaeorrhoea* but when crystals, isolated from the crystalliferous strain, were added to the spores of the acrystalliferous strain, 100 per cent mortality resulted in 6 days. Vaňková concluded that the crystals "merely induced germination of the spores in the intestine of the caterpillar which results in septicemia." Vaňková noted that the gut ceased to function; however, she did not take pH readings in the gut after crystals were fed. In the functioning gut of the feeding insect, buffers are continually produced and conditions inhibitory to germination of spores are constantly maintained. The crystal toxin harms the gut, which stops functioning, relaxes in paralysis, and ceases secretion. Conditions change, the pH falls, and germination of spores takes place. The primary and most important action, however, is the effect of the toxin on the gut (9, 53).

5. Phospholipase activity and other effects. What then are the secondary effects of these bacteria and how important are they in killing insects? Toumanoff and Vago stated that young cultures (presumably composed mainly of vegetative cells) are capable of killing a few silkworm larvae when fed (113). Vaňková reported 90 per cent mortality of *E. phaeorrhoea* in 8 days after feeding

TABLE 5*

Comparison of Bacillus cereus lecithinase and Clostridium perfringens lecithinase

	B. cereus Lecithinase	*Cl. welchii* Lecithinase
Lecithinase activity		
Ca requirement	+	+
Optimal pH	6.8–7.2	7.0–7.6
Thermostability, %:		
60 C 10 min	82	87
Boiling 10 min	32	45
Resistance to oxidation	+	+
Inactivation by formalin	+	+
Precipitation by ⅔ saturated $(NH_4)_2SO_4$	+	+
Activity on egg yolk lipoproteins	++	+
Inhibition by normal serum proteins	+	−
Biological activity		
Egg yolk reaction and Nagler reaction	+	+
Hemolytic activity	+	+
Inhibition of hemolysis by normal serum	+	−
Dermonecrotic effect in guinea pigs	+	+
Toxicity to mice	+	++

* After Chu (28).

on vegetative cells of *B. thuringiensis* var. *thuringiensis* (Czech strain) (119). Is it possible that other toxic substances are produced by certain strains of these bacteria?

In 1953, Toumanoff reported on a study of the phospholipase C (lecithinase D) production by various members of the genus *Bacillus* (112). He confirmed Colmer's (29) original statement that only those bacteria of the "*B. cereus* group" produce phospholipase C. As did Chu (28), he pointed out the resemblance of the *B. cereus* phospholipase to the α-toxin produced by *Clostridium perfringens* (see table 5) and concluded, "It is not to be excluded that the production of lecithinase plays a role in the toxic effect of these bacilli of the *cereus* group on insects." In 1954, Toumanoff found that broth filtrates of *B. thuringiensis* var. *alesti* were practically innocuous by injection in wax moths and were harmless when given *per os* (114). However, when he precipitated protein from broth culture filtrates by Chu's method with ammonium sulfate (28), (thereby concentrating the phospholipase C), he found the precipitate was toxic both by injection and *per os*. He concluded that phospholipase C contributes partly to the toxic action of *B. thuringiensis* var. *alesti*.

At the same time Heimpel (45, 47, 48) showed that strains of *B. cereus* that produced relatively large amounts of phospholipase C were more virulent for larch sawfly larvae (*Pristiphora erichsonii*) than were strains producing lesser amounts. These experiments were confirmed by Kushner and Heimpel (62). Other species of the genus *Bacillus* that did not produce this lecithinase were found to be nonvirulent for this insect. Heimpel (45) showed that *B. thuringiensis* produced phospholipase C in approximately the same relative amounts as his pathogenic strains of *B. cereus*, and later stated that all crystalliferous bacteria (with the exception of *B. entomocidus* var. *entomocidus* and *B. entomocidus* var. *subtoxicus*) produced phospholipase C in relatively high amounts (48). He showed that the larch sawfly has a relatively low pH in the midgut (pH 7.0 to 8.4), which not only permits good growth of *B. cereus* but is also well within the pH range (6.8 to 7.4) for phospholipase activity. He pointed out that most *Lepidoptera* are immune to *B. cereus* because of the restriction on growth by the high midgut pH, but also emphasized that stress in the form of starvation lowered the pH, allowing good growth of *B. cereus* or crystalliferous vegetative rods and concurrent production of phospholipase C with its inevitable results. A paper by Bonnefoi and Béguin (17) recently confirmed these results. Stress, capable of increasing susceptibility to disease in insects, has been discussed by Steinhaus (100, 101).

Thus we find that growing cells of most of the crystalliferous bacteria produce a toxic enzyme, phospholipase C, capable of breaking down insect lecithins. If there are changes in the midgut of normally resistant *Lepidoptera* (and these changes are possible) this toxic substance almost certainly harms the insect.

A third toxic substance has been demonstrated by McConnell and Richards (74). This material, produced by *B. thuringiensis* var. *thuringiensis* cultures *in vitro* or *in vivo*, is detectable after 24 hr incubation and withstands autoclaving at 15 pounds for 15 min. The substance is water soluble and dialyzable but is not toxic to the insects tested *per os*. The material must be injected to kill and requires 4 days or more to cause mortality. Production of the toxic material in liver broth begins after some 8 hr incubation on a shaker at 30 C, and reaches its maximum concentration in approximately 24 hours. Apparently the toxin production is in no way associated with sporulation. McConnell and Richards showed that a strain of *B. cereus*, which they state "... to be non-pathogenic for insects", produced a similar toxic material but apparently in lesser concentration (74). They conclude that the heat-stable toxin may play only a minor role in insect disease since it is produced also by a "non-pathogen." Some *B. cereus* strains, however, are pathogenic for several insects (*e.g.*, larch sawfly and codling moth). If the heat-stable toxin is only effective in the blood, then its effect should be determined in insects that have been demonstrated to be susceptible to *B. cereus*. McConnell and Richards tested this "*B. cereus* group" material against *G. mellonella*, the American cockroach *Periplaneta americana*, and *Aedes aegypti*. Neither *B. thuringiensis* nor *B. cereus* are pathogenic for any of these insects *per os*, so McConnell and Richards may have discovered something of interest concerning the mode of action of *B. cereus* strains which should be investigated further.

6. Histopathology. Histological investigations have added to our knowledge of the possible action of some of these toxic substances. Berliner pointed out that *B. thuringiensis* var. *thuringiensis* spores germinate and grow very soon after in-

gestion by *A. kuehniella* larvae (16). This is to be expected as this insect has a midgut pH below 8.4. As growth proceeds in the gut, eroded areas develop particularly at the posterior end of the midgut where fragile cells link the midgut and hindgut. Berliner stated that it was through these eroded areas the bacteria invaded the hemocoele, causing a fatal septicemia.

Mattes presented another hypothesis. Like Berliner he claimed that the bacteria multiplied extensively in the gut before the midgut epithelium showed signs of breaking down (72). He stated that the type of erosion noted by Berliner was encountered infrequently and suggested that the bacteria entered the blood by moving between the cells. He explained the infrequent observation of this phenomenon as due to very rapid migration of the bacteria. Heimpel pointed out that this type of invasion was never encountered in *A. kuehniella*, although the mode of invasion described by Berliner was seen frequently in a histological study designed to repeat Mattes' work (45).

Too much weight placed on the results of the histopathology of *A. kuehniella* infected by crystalliferous bacteria would at this time be unwise, for Heimpel and Angus have indicated that this insect apparently responds to the disease in a different manner than do type I and type II insects (53). There is no general paralysis and to date it has not been demonstrated that the gut becomes paralyzed. If the *B. thuringiensis* var. *thuringiensis* crystals are freed from spores and are fed, the resulting mortality is only a fraction of that realized when crude cultures containing spores and crystals are fed. Heimpel showed that there is little destruction of the mudgut epithelium while the bacteria are growing in the gut but that there is a tendency for the epithelial cells to loosen one from another, and as described by Mattes the distal ends of the epithelial cells lose their ability to take stain. The weakest point of the gut is apparently the junction of the midgut and the hindgut where fragile cells connect the two organs (45).

Tanada stated that *Pieris rapae* L. (midgut pH from 6.4 to pH 8.1) ceased feeding shortly after ingesting spores of *B. thuringiensis* var. *thuringiensis* (107). This is usually indicative of gut paralysis (53) and this would class *P. rapae* as a type II insect. Tanada showed that the anterior midgut epithelium begins to disintegrate shortly after spores (and crystals) of *B. thuringiensis* var. *thuringiensis* are ingested; few vegetative rods are in evidence. The bacteria multiply but no excessive numbers of bacteria can be observed in the gut lumen. The bacteria penetrate the muscle layer into the blood where an intense septicemia takes place, eventually killing the insect. This description of the histopathology of the disease in *P. rapae* is typical for the type II insect.

Toumanoff and Vago studied the histopathology of the infection of silkworm larvae by *B. thuringiensis* var. *alesti* and pointed out that if fed very small amounts of the crude culture, the silkworm showed symptoms indistinguishable from those of the type II insects; however, larger doses of culture caused general paralysis and death in 1 to 2 hr (113). They showed that there was a very rapid degeneration of the midgut, and postulated that the sporulated culture contained a toxin. They were not aware at this time of the presence of the toxic crystal, but pointed out that the spores did not have time to germinate before the insect was paralyzed and died. They stated that the epithelial cells soon lost their cohesion and formed a spongy degenerating mass. When fed in small doses, the bacteria germinated and multiplied somewhat slowly in the gut lumen and faster after the epithelial cells had begun to separate, allowing the bacteria to penetrate to the surrounding muscle layer. Toumanoff and Vago, however, did not note that the gut of larvae fed small doses was paralyzed. Heimpel and Angus showed that by feeding spores and crystals of *B. entomocidus* var. *entomocidus* to silkworms, gut paralysis was evident twenty minutes after ingesting toxin as shown by the relaxed appearance of the circular muscle around the gut (53). Since *B. entomocidus* var. *entomocidus* does not produce phospholipase C, the histopathological changes seen in the gut can be attributed to the action of the crystal toxin. These are general loosening of the cells one from another, and from basement membrane, relaxation of the gut musculature, degeneration of the epithelial cells and eventually degenerative effects such as fenestration and "fraying" of the body muscle in the hemocoele.

Heimpel and Angus (53) stated that the crystal alone causes gut paralysis, which is the primary and most important effect of this bacterium on the insect. They postulated that this cessation of gut function is caused by the rapid dissolution of cell-cementing substances which exposes the cells to the action of gut contents,

causing autodigestion of the cells from the disorganized tissue. This still does not explain why the toxin affects the type I insect so as to cause complete gut permeability (as in the silkworm), whereas type II insects show rapid epithelial damage but no leakage of the gut contents into the blood.

Perhaps the answer lies in the direction taken by Pipa and Cook (86) and Richards and Schneider (88) in their recent studies of the connective tissue of the louse, the cockroach, and the silkworm moth. According to these researchers, the connective tissue surrounding the organs within the hemocoele, and intimately connected to the neurolemma and the basement membrane, is composed in part of neutral or acid mucopolysaccharide and protein. Richards and Schneider point out that the bulk of the neurolemma is composed of a protein that is obviously different from collagen, elastin, and reticulin, with optical properties that imply the presence of lipid. There is no clear statement in the literature as to the composition of the cell-cementing substances; however, Pipa and Cook state that the connective tissue in the louse shows many of the properties of a mucoprotein, glycoprotein, or neutral mucopolysaccharide, and it is quite possible that the cell-cementing substances, which histologically appear to be continuous with the basement membrane, are composed of the same or very similar materials. Histological studies leave no doubt that the cells separate and come away from the basement membrane at an early stage in the progress of the disease, in both type I and type II susceptible insects. This places us on familiar ground, bacteriologically speaking, since we are aware of the effect of vertebrate pathogens, capable of producing hyaluronidase, upon vertebrate cell-cementing substance. In this connection Day and Powning (30) demonstrated that gut epithelial cells from the German cockroach *Blattella germanica* (L.) can be separated *in vitro* by pig-testis hyaluronidase. This is not to imply that hyaluronic acid is the main component of insect cell-cementing substances, but suggests that a similar substance, possibly a mucopolysaccharide of some sort, is present in this material. It is possible to postulate that the bacterial crystalline protein is the precursor of an enzyme, which under suitable conditions in the insect gut attacks a substrate in the substance cementing the epithelial cells. It is equally possible that this enzyme also affects the cell membrane, since rapid breakdown of epithelial cells is common in most of the susceptible insects examined.

7. Site of action. Heimpel and Angus established that the primary site of action of the crystal toxin in silkworm larvae is the anterior third of the midgut (53). Heimpel stated that the contents of this portion of the gut have a pH of approximately 9.8 (55). As we have stated previously, this pH is not high enough to dissolve the crystal unless a reducing agent is present, and of course the silkworm gut contents are strongly reducing. There are *Lepidoptera* such as *Choristoneura fumiferana* (Clem.) in which the pH in the anterior gut is approximately 8.5 and it is unlikely that the crystal would dissolve readily at this pH. An alternative hypothesis to explain the quick release of the toxin is that proteases in the insect gut release a smaller, toxic molecule. Heimpel and Angus have reported a protease in the silkworm gut capable of breaking down the crystal protein, and they suggest that insects not susceptible to the crystalliferous bacteria may lack such enzymes (53). In such a system, the inert crystal could be thought of as a pro-toxin.

D. Standardization of the Toxin

In 1949, when Steinhaus began his studies on the effect of *B. thuringiensis* var. *thuringiensis* against the alfalfa caterpillar, the bacterium was considered a disease organism and estimates of its concentration were based on the number of spores present. Fortunately, Steinhaus grew his organism on nutrient agar, which allows the formation of a crystal in every sporangium; a spore count would thus give an approximate crystal count. Due to some inevitable mortality of spores and to clumping of spores, the actual crystal count must have been higher than the spore count. However, when mass production of the organism was started in Czechoslovakia, France, and the United States, it soon became obvious that the spore count was not an accurate measurement of the virulence of the bacterial preparation. The reason for this was the variable response in sporulation and crystal formation in the type of media employed in large commercial fermentors. Actually, in the fermenting industry growth conditions can be controlled to a remarkable degree. The factors that govern the production of the maximal number and proper quality of crystals cannot be measured by counting spores. Neither can the crystal toxicity be measured by a

microscopic count (in itself a tedious procedure). Heimpel and Angus described a bacterium, *Bacillus entomocidus* var. *subtoxicus*, which produces as many crystals as, and is morphologically identical to, *B. entomocidus* var. *entomocidus*, yet the latter is at least 100 times more toxic for the silkworm than is the former. In other words, the presence of the crystal is still not a reliable measure of toxicity (52).

The French workers under the direction of Grison realized this fact early in their work and in a series of papers described a biological testing method which measures the toxicity of the preparation. In 1956, Lemoigne *et al.* developed a spray apparatus (65), described in more detail by Burgerjon (23), which allowed them to spray a suspension of spores and crystals on a flat surface so that the deposit was reasonably constant. Burgerjon described a method of testing *Pieris brassicae* L. on pieces of sprayed cabbage leaf (7.5 by 12.5 cm) retained in plastic boxes (24). The treated leaves were removed after 48 hr and were examined for area consumed. This criterion, the amount of feeding, was inversely proportional to the dose placed on each leaf. Mortality varied with temperature and larval stage.

Using Burgerjon's techniques Bonnefois *et al.* (18) and Burgerjon (25) tested *B. thuringiensis* against a large number of *P. brassicae* under standard conditions and obtained an LD_{50} curve by probit analysis. They then retained this bacterial preparation as a standard and proceeded to set up a system of measuring the virulence of subsequent *B. thuringiensis* material produced.

The procedure was as follows. Various weights of the standard *B. thuringiensis* preparation were each placed in 10 ml of water. Various weights of the unknown preparation were similarly diluted. These preparations were fed to standard size *P. brassicae* larvae reared under controlled conditions (25 C and 75 per cent relative humidity). The weight of standard material giving an LD_{50} of test insects was divided by the original LD_{50} standard weight from the probit analysis and this fraction was multiplied by the LD_{50} weight of the unknown material. This procedure reduces the possible error introduced by using different batches of insects. The result is an "adjusted" LD_{50} weight of unknown material which is then divided into 10,000 (milliliters of water used to prepare the suspension). This gives a dilution factor in milligrams of water per milligram of unknown which provides a suspension approximately equal in virulence to the standard material used in the original probit analysis. It is a most useful method, as the dilution factor can be taken from the testing laboratory to the field and used directly to prepare material for field application. An attractive feature of the method is that it accommodates for variability in different insect rearings and also could be used between different insects species. The result obtained is more practical than a system based on unit-weight comparison.

One might be tempted to question the necessity of an involved test for toxicity when a spore count could be used to measure the bacterial content and, by interpolation, the crystal count. According to Brown *et al.* (20), there are strains of *B. cereus* which have latent *Bacillus anthracis* characteristics and if such strains should contaminate a crystal-forming culture at any stage in the manufacturing process, a spore count and a casual microscopic examination of culture would not be sufficient to detect the presence of the contaminant. However, a biological test for toxicity would indicate a reduction of virulence which would indicate the need for a more extensive examination of the preparation.

The French standardization method is the only published attempt in this direction. Other systems, such as a biological unit based on the weight of a standard preparation necessary to give an LD_{50} in test insects, have been proposed and are probably in use now but we feel that the French system is more readily applicable and more flexible than any other suggested to date.

IV. Field Application

Steinhaus pointed out that most bacteria that are insect pathogens are usually harmless to animals and plants (98). After Brown *et al.* (20) had reported their experiments on the selection of pathogenic strains of anthrax-like bacteria from *B. cereus* cultures, Steinhaus sent Brown most of the known crystalliferous bacterial varieties and these were examined for the possibility that clones of pathogenic organisms might exist in the crystalliferous cultures. Steinhaus reported that Brown found no evidence of pathogenic strains (102). The question of the safety of these bacteria was finally resolved by Fisher and Rosner, who reported that ingestion and inhalation tests showed that *B. thuringiensis* var. *thuringiensis* was harmless to warm blooded animals (32) Thousands of pounds of these bacterial prepara-

tions have been used in the field on a wide variety of crops without a single report of damage to the protected plants. Finally, a report from Germany and one from France indicate that bees foraging within a sprayed area would not likely be harmed by the bacteria (60, 63).

A large number of tests have been conducted since 1950 in many countries throughout the world. This has been made possible by the production of the crystalliferous bacteria on a limited commercial scale in Czechoslovakia, France, Germany, and by several companies in the United States.[7]

A detailed account of the field trials would be more appropriately placed in an entomological journal.[8] As a general statement, it can be said that over a hundred species of *Lepidoptera* are susceptible to the crystalliferous bacteria. Some taxonomic groups (*e.g.*, the family *Geometridae*) appear to be less susceptible (54, 75). Sawflies and bees (*Hymenoptera*) are apparently unharmed by the crystal toxin (60, 63) but some sawflies are susceptible to bacterial infection by the spores (48). Some *Diptera* have been demonstrated to be susceptible to *B. thuringiensis* var. *thuringiensis* (41) and this is a most interesting point to those concerned with mode of action. It is tempting to speculate that most dipterous larvae, although possessing digestive tracts in which the pH is usually acid to slightly alkaline, regurgitate an alkaline digestive juice containing proteases into the surrounding nutritive medium. In this way they may break down the crystal to a toxic substance outside the body and then ingest it with the dissolved food. A low order of susceptibility has been noted in some species of *Coleoptera* (96). Although other groups of insects have not been as extensively studied it appears that this microbial insecticide is most effective against *Lepidoptera* larvae.

[7] The North American formulations have been released on an experimental basis under a number of trade names: "Thuricide" (Stauffer Chemical Company and Bioferm Corporation); "Larvatrol" (Nutrilite Products, Inc.); "Agritrol" (Merck and Company, Inc.); and an unnamed preparation of *Bacillus thuringiensis* released by Rohm and Haas Company. The European formulations have been prepared by various governmental agencies.

[8] For reference to field trials using crystalliferous bacteria against a variety of insects since 1957, the following papers are worthy of attention: 19, 22, 26, 27, 36, 38, 41, 61, 69, 70, 80, 85, 106, 108, and 118.

V. Concluding Remarks

Certain areas need further investigation. From informal talks with representatives of the manufacturing firms we have obtained the impression that there is little difficulty in growing the crystalliferous bacteria with a good yield of toxin. Many producers then extend the bacteria with materials such as bentonite, clay, and the like. This provides a preparation that is excellent when used as a dust. Although a dust application is more effective than a water spray in certain climates (37, 39), water sprays appear to be more effective on similar crops under other conditions in the Northeastern United States (75). Some of the materials used to extend the bacteria do not remain in suspension when mixed in water and their settling can complicate spraying operations. Forest spraying requires high-concentration, low-volume sprays and this necessity has favored the use of oil carriers, but unfortunately many clay-extended preparations are not suitable for use with oil. Consequently, much research must be applied to the problem of preparing unextended preparations of these bacteria for use in specific spray mixtures.

There is little doubt that the crystal produced by these bacteria is the active agent in the insect and consequently intensive work is needed to standardize this toxic substance. Unfortunately we still do not know the action of the toxin and thus it is nearly impossible to derive a quantitative test *in vitro* to measure the titer of a preparation. It would seem advisable then to concentrate on a biological test for the present and spend considerable effort in determining the precise action of this substance on the insect. Once the substrate has been discovered, it should not be difficult to develop an appropriate method of measuring the extent of action of a given concentration of toxin.

It appears that we have in the crystalliferous bacteria an extremely useful weapon that we may use against noxious insects of the orders *Lepidoptera* and *Diptera*. Naturally it is not to be considered the final answer to all insect problems in this direction. However, when wisely used these bacterial preparations should be highly effective.

There is a tendency for some of the investigators who have used them to compare them with chemicals that are currently in use. Accordingly, the weights used are carefully compared with the weights of highly toxic chemicals applied, even though there is no need (as far as we are aware)

to restrict the use of the known bacterial insecticides. The care necessary in applying the deadly chemicals relates directly to residue problems and this points up one of the desirable attributes of the bacterial insecticide. It is highly specific for many noxious insects but apparently does not harm their parasites and is innocuous for bees and warm-blooded animals that might contact the organism in the sprayed area.

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