THE HISTOPATHOLOGY AND TOXICOLOGY OF *BACILLUS THURINGIENSIS* BERLINER ON CERTAIN LEPIDOPTERA LARVAE

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ABSTRACT

THE HISTOPATHOLOGY AND TOXICOLOGY OF BACILLUS THURINGIENSIS BERLINER ON CERTAIN LEPIDOPTERA LARVAE

by M. Ephatha A. Materu

Studies on the toxicology of a commercial preparation of Bacillus thuringiensis Berliner were made on the greater wax moth, Galleria mellonela (Linnaeus), by feeding larvae of various ages with a treated diet.

Galleria larvae were susceptible to Bacillus thuringiensis but there were apparent differences in mortality for different ages and dosage levels. Even if treated larvae did not die quickly, they did not increase in size.

The oxygen uptake of treated Galleria larvae, as determined with a Warburg respirometer, was found to be lower than that of untreated larvae.

Mediterranean flour moth larvae, Anagasta (= Ephestia) kühniella (Zell.), were also fed on diets treated with varied amounts of B. thuringiensis. Due to the unselected nature of the test insects and the small number of the insects available the results were not conclusive, except that the Mediterranean flour moth was demonstrated to be less susceptible than Galleria to B. thuringiensis.

Histopathological studies were made on Galleria larvae treated with B. thuringiensis. Larvae were allowed to feed on a treated diet

for a period of time. At intervals the larvae were removed from the treated diet, fixed, sectioned and the structure of mid-gut studied microscopically. No spores were visible in the body but alteration and disintegration of the mid-gut epithelium of treated larvae was evident.

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INTRODUCTION

In recent years there has been great concern among entomologists about resistance of insects to insecticides and as a result there has been experimentation with other methods of insect control. Among such methods is the use of certain bacterial pathogens. The better known bacteria for this purpose fall into the Bacillus group, and the best known is B. thuringiensis Berliner. B. thuringiensis was first isolated and described in Germany from dying larvae of Mediterranean flour moth Anagasta (= Ephestia) kühniella (Zeller), by Ernst Berliner in 1911 (Berliner, 1915). The isolated bacterium was described as an anaerobic, slightly motile, compact, gram-positive, straight spore-forming bacillus with firm membrane, and easily stained with aniline dyes. The length of the spore is approximately 5 μ, with thickness varying from 1 to 8 μ. They occur in chains of 3 to 4 cells, though seldom of filamentous length. The spore itself was described by Berliner as 2 μ long and 1 μ wide with the spore occupying one end of the sporangium with a characteristic 'chrystalline inclusion' ("Restkorper" or "residual body") at the other end. The "Restkorper" was observed to cling to the spore when it is discharged from the sporangium. From available data (Heimpel and Angus, 1956) it appears as though both the spore and the 'crystalline inclusion' are apparently necessary for insecticidal activity in some insect species, while either one alone is sufficient in others. The spores grow well on artificial media and production of spores is very rapid on solid media and in well aerated submerged broth fermentations.

B. thuringiensis spore preparations are stable and when stored under dry conditions can remain active for a number of years. Available data show that spore preparations are not adversely affected by chemical insecticides (Heimpel and Angus, 1956).

Though B. thuringiensis was known to be toxic to insects for a long time its possible use for insect control was not studied until members of the International Corn Borer Institute reported its effectiveness against the European corn borer, Ostrinia (= Pyrausta) nubilalis (Hübner), (Husz, 1928, 1929; Metalnikov and Chorine, 1929). This report prompted field studies using laboratory prepared spores. Since there was no method yet for large scale production of this bacterium, and due to inconsistencies in the results and failure to duplicate earlier studies together with the onset of cheaper and effective insecticides such as DDT, (1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl) ethane), this subject was again dropped. The development of insect resistance to DDT and some of the organo-phosphorous insecticides along with reports of control of the white grub with preparations of B. popilliae (Dutky) again stimulated research with B. thuringiensis.

The action of B. thuringiensis has been studied and reported for various insect species and even Annelid worms. B. thuringiensis has been found most effective on lepidopterous larvae but it has also been found to kill larvae of other orders. Among the reports on the action of this bacteria are those by Steinhaus (1951a, 1951b, and 1954) on alfalfa caterpillars, Colias eurytheme Borsduval; Hall and Andres (1959) on cabbage looper, Trichoplusia ni (Hbn.); Grigarick and Tanada (1959) on cabbage looper; Kantack (1959) on Indian-meal moth, Plodia interpunctella (Hbn.); Tanada (1956) on cabbage worm, Pieris rapae (L.);

De and Konar (1955) on khapra beetle, Trogoderma granarium Everts; Hall (1955) on western grape leaf skeletonizer, Harrisina brillians B. and McD.; Hall (1954) on sod webworm; Metalnikov and Chorine (1929), McConnell and Cutkomp (1954), Chorine (1929), Husz et al. (1928), on the European corn borer, Ostrinia (= Pyrausta) nubilalis (Hbn.); Burgerjon and Klinger (1959) on Tortrix viridina (L.); Vasiljevic (1957) on Hyperantria cunea (Drury); Heimpel (1955) on larch saw fly, Prisphora erichsonii (Htg.); Splittstoesser and McEwen (1961) on cabbage looper, Trichoplusia ni (Hbn.); Borgatti (1961) on house fly, Musca domestica L. and Smirnoff and Heimpel (1961) on earthworm, Lumbricus terrestris L. Other investigators such as Hall and Dunn (1958); Heimpel and Angus (1956 and 1958); Rabb and Steinhaus (1957); Steinhaus and Bell (1953); Tanada (1953); and Tapley and Materu (1960) have also studied the toxicity of bacillus on insect larvae. For a detailed review the reader is referred to the excellent reports by Steinhaus (1956) and Tanada (1959).

In 1957 and 1958 commercial quantities of dried spore preparations were produced by pilot-plant operations. This prompted many investigators to try field studies with the bacteria both in dust and wettable powder formulations. The results of many of these trials were disappointing. This was probably due to general lack of knowledge of the behaviour of the bacillus under field conditions and also lack of sufficient preliminary laboratory data on effective dosage levels.

There was a need to assay the toxicity of the bacillus and for this work easily cultured insects under laboratory conditions were needed. Galleria mellonella (L.) has become a well known laboratory insect and its life cycle and laboratory rearing conditions were well

known (Beck, 1960). It was therefore thought that Galleria would be a suitable test insect for general toxicological and bioassay studies.

The present study is a preliminary investigation undertaken to study (a) the effectiveness of B. thuringiensis against larvae of the greater wax moth when incorporated in the larval diet, (b) effective dosage levels which can bring about mortality of the larvae, (c) effect of the bacillus on the larval metabolism in terms of respiration, (d) histopathological changes in the larval alimentary tract, particularly the mid-gut, and (e) to compare the relative effective dosage levels on other insect larvae.

THE TEST INSECTS

The insects used in these experiments were mainly larvae of the greater wax moth and the Mediterranean flour moth. The larvae were raised in the laboratory. The stocks were a random selection and there were noticeable variations in size and rate of growth. Wax moth is an insect of world-wide distribution, and is occasionally a serious pest in apiaries. The larvae feed on the waxy brood combs and pollen stores of the honey bee, Apis mellifera L. The adult moths were put into a large glass jar in which a pleated wax paper was added. The jar was put into a closed incubator at 30° C. Each morning the eggs which were oviposited on the wax-paper were removed and incubated in a petri-dish in an incubator at the same temperature. After 5 days the eggs were ready to hatch, and were transferred to a half pint jar to which the larval diet had been added.

The larval diet was made according to Beck (1960) in the following ratios (Table 1):

TABLE 1.--Artificial dietary medium for laboratory rearing of the greater wax moth, Galleria mellonella (L.)

Constituents	Amount (G.)	Concentration (Mg./G.)
Honey, strained	25.0	236
Glycerol	22.0	207
Water	10.0	94
Pablum, mixed cereal	34.0	322
Brewers yeast powder	10.0	94
Bees wax, yellow	5.0	47
Total	106.0	1,000

The wax was melted in a glass jar and dissolved in ether. This was added to a mixture of pablum and yeast to which a little ether had been added. This diet was again well mixed and the ether evaporated off. The liquid components were mixed together in a glass jar and then added to the pablum-yeast-wax mixture. This was blended together and left in the incubator at 30° C. for 24 hours before it was used.

The Mediterranean flour moth stock was fed on a mixture of fine wheat flour (95%) and brewers yeast (5%). The moths were put in a big jar containing this diet and oviposition took place in the diet and the larvae when they hatched fed on the diet. Mediterranean flour moth larval growth was relatively slow compared with the growth of wax moth larvae.

TOXICOLOGICAL TESTS WITH *BACILLUS THURINGIENSIS* ON *GALLERIA* LARVAE

(a) Procedure

In these tests *B. thuringiensis* spore preparation, Thuricide Wettable Powder (Stauffer Chem. Co.), with a spore count of 50.5×10^9 spores per gram was used. A known weight of the spore powder was mixed completely in the liquid parts of the larval diet (honey, glycerol and water) which were then thoroughly mixed with the solid part of the diet and kept for 24 hours before the larvae were fed on it. Fifty-three grams of diet were used in each trial. An untreated diet made in the usual way was used as a control.

Three age groups of larvae were used in these experiments: 7-day-old larvae (4th instar), 13-day-old larvae (app. 5-6th instar) and 16 days old and over (7th instar).

The dosage levels used in these trials were 1, 2, 2.5, 3, and 4 grams in 53 grams of larval diet and the temperature was kept at 30° C. in the incubator.

In a group of the same age, it was usual to find that the larvae were of different sizes due to variations mentioned earlier. In these experiments therefore, larvae of the same age and approximately the same size were used. About 5-7 grams of treated diet was put on a petri dish, 10 larvae introduced, covered with a glass cover, labeled and put in an incubator at a constant temperature of 30° C. Counts in the first two groups were made at intervals of 24 hours, but in the

last group (i.e. mature larvae) counts were made at 3 day intervals. Each treatment had two replicates so that 20 larvae were used for each dosage. The controls using untreated diet were also replicated in the same way.

The results of these experiments are summarized in Figures 1-6.

(b) Results

Figures 1 and 2 show that there was a considerable variation in the results. For 7-day-old Galleria larvae, 2.0-4 grams of bacillus spore formulation in 53 grams of diet effected 50% kill by the second day, while 1.0 gram of spore formulation killed 50% by the sixth day. With 13-day-old larvae however, 2.5 to 4.0 grams of bacillus spore killed 50% of larvae at the end of the second day and by the fourth day all dosage levels had killed 50% or more.

The same dosage levels had little effect on the mature larvae. None of the dosages killed 50% of the larvae in this experiment and most of them emerged as adults.

With the 7-day, 13-day, and mature larvae as can be seen in the figures, sometimes lower dosages killed higher percentages of larvae. Whether or not this was due to variations in the larvae or variations in the bacterial activity is not known.

In the three experiments as is apparent from figures 1-6, there was an initial high kill followed by a relatively lower kill. This is shown by the near-plateau nature of figures 1 and 2. It is also observed that the older the larvae the more time the same bacillus concentrations take to kill. For example, while 2 grams of bacillus per 53 grams of diet caused 50% kill in less than 48 hours to the 7-day-old larvae, the same mortality was effected in 13-day-old larvae at 4 days.

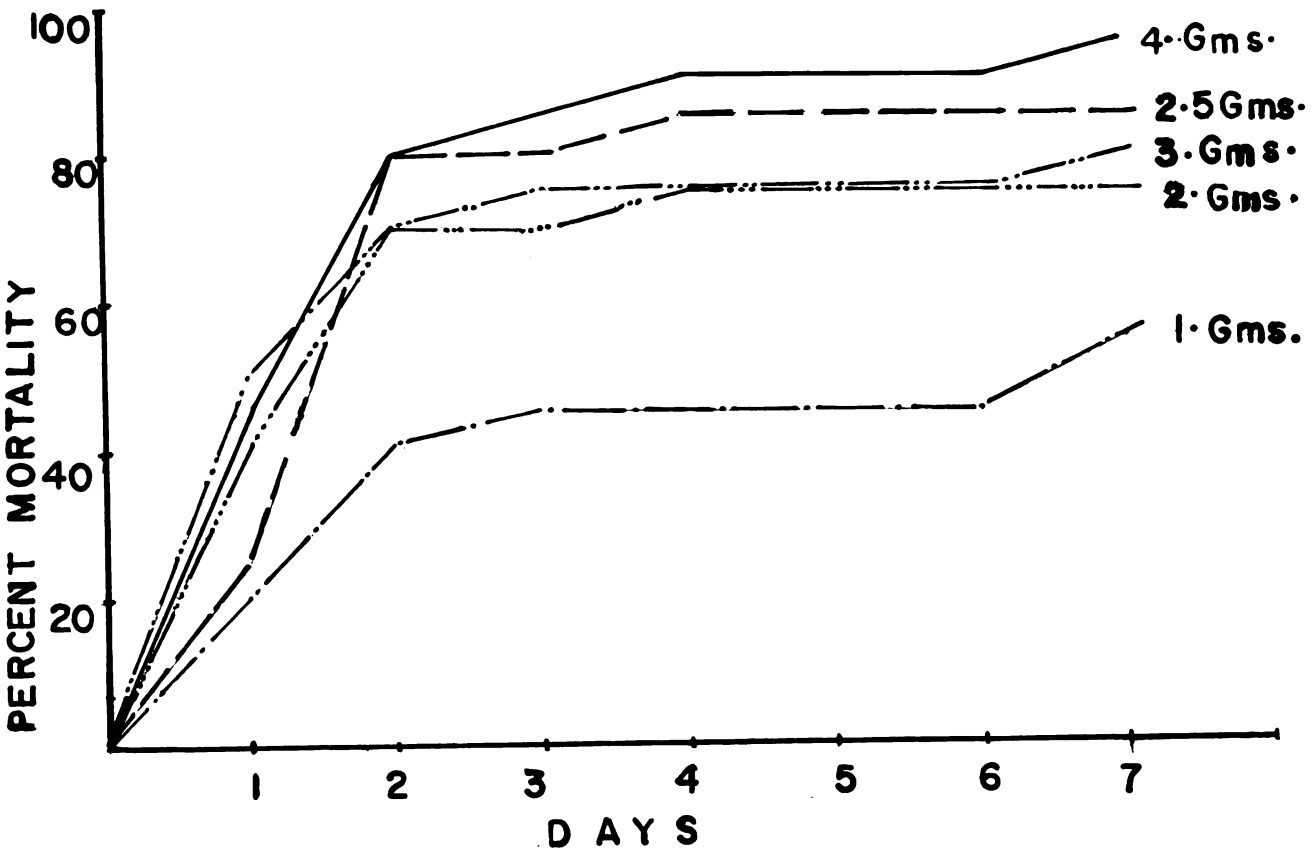

Fig. 1.--Seven-day-old Galleria larvae fed on various dosages of B. thuringiensis.

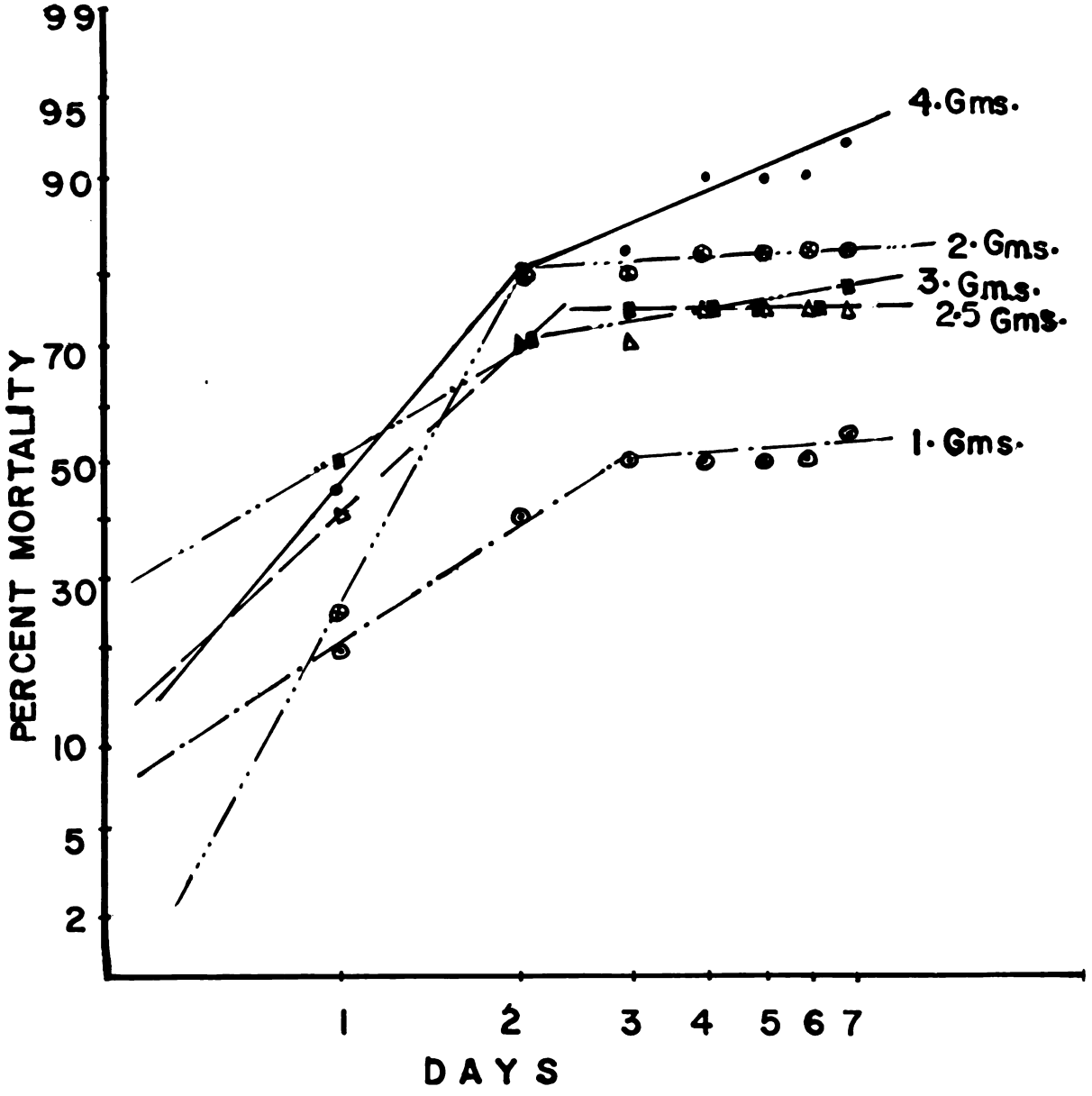

Fig. 2.--Mortality of 7-day-old Galleria larvae fed on various dosages of B. thuringiensis. (Log-dosage-probit transformation.)

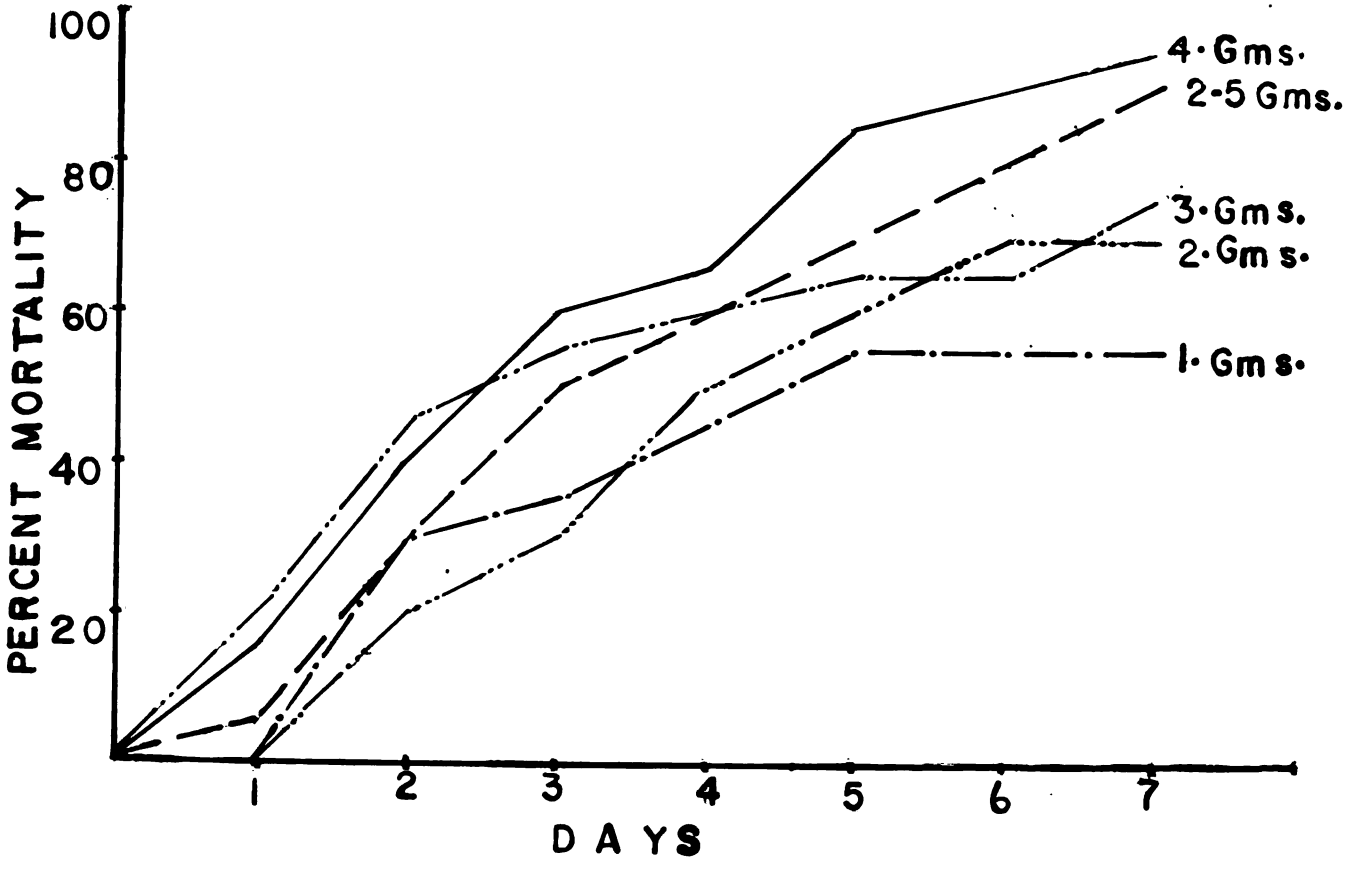

Fig. 3.--Thirteen-day-old Galleria larvae fed on various dosages of B. thuringiensis.

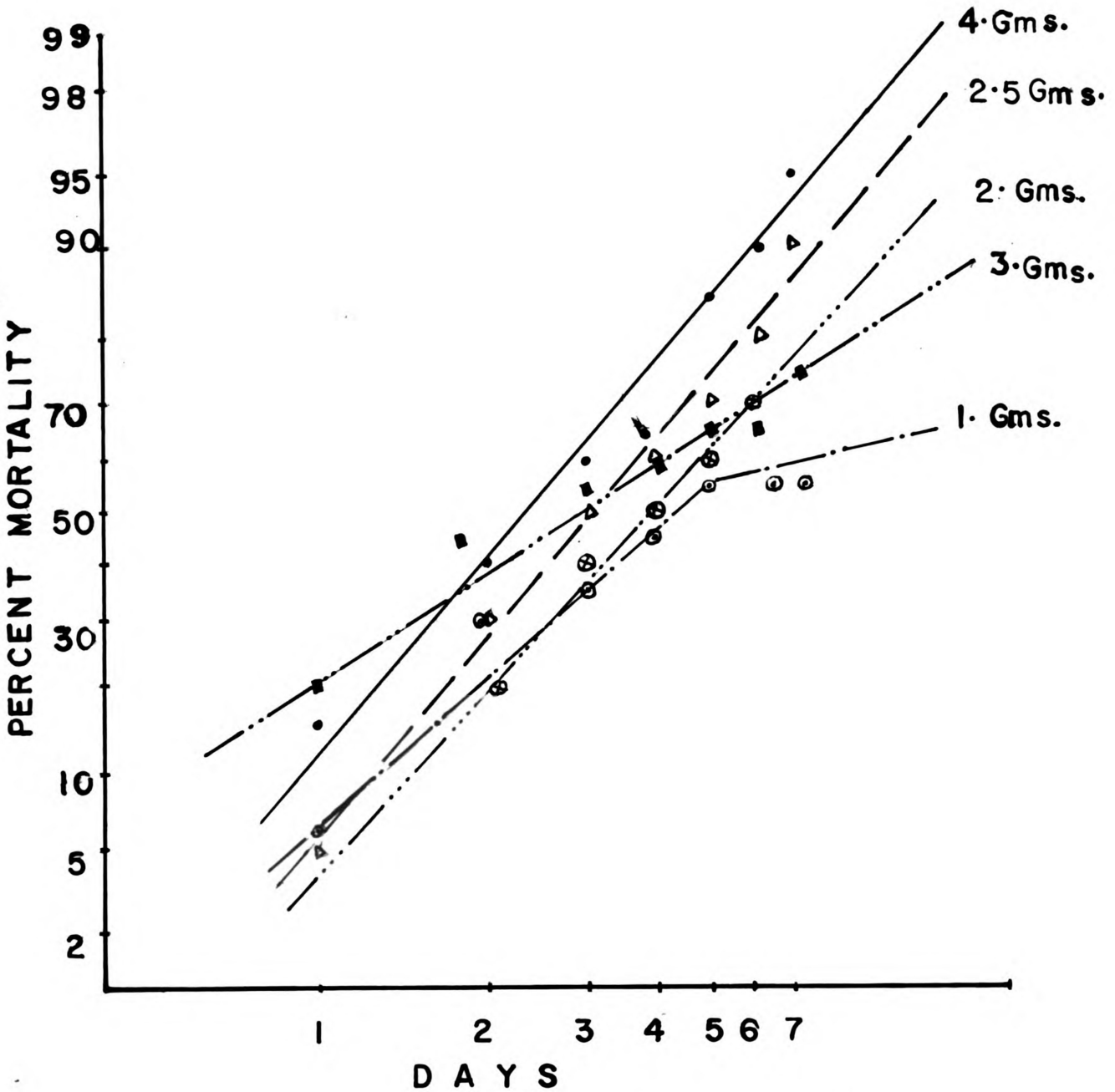

Fig. 4.--Thirteen-day-old Galleria larvae treated with various dosages of B. thuringiensis. (Log-dosage-probit transformation.)

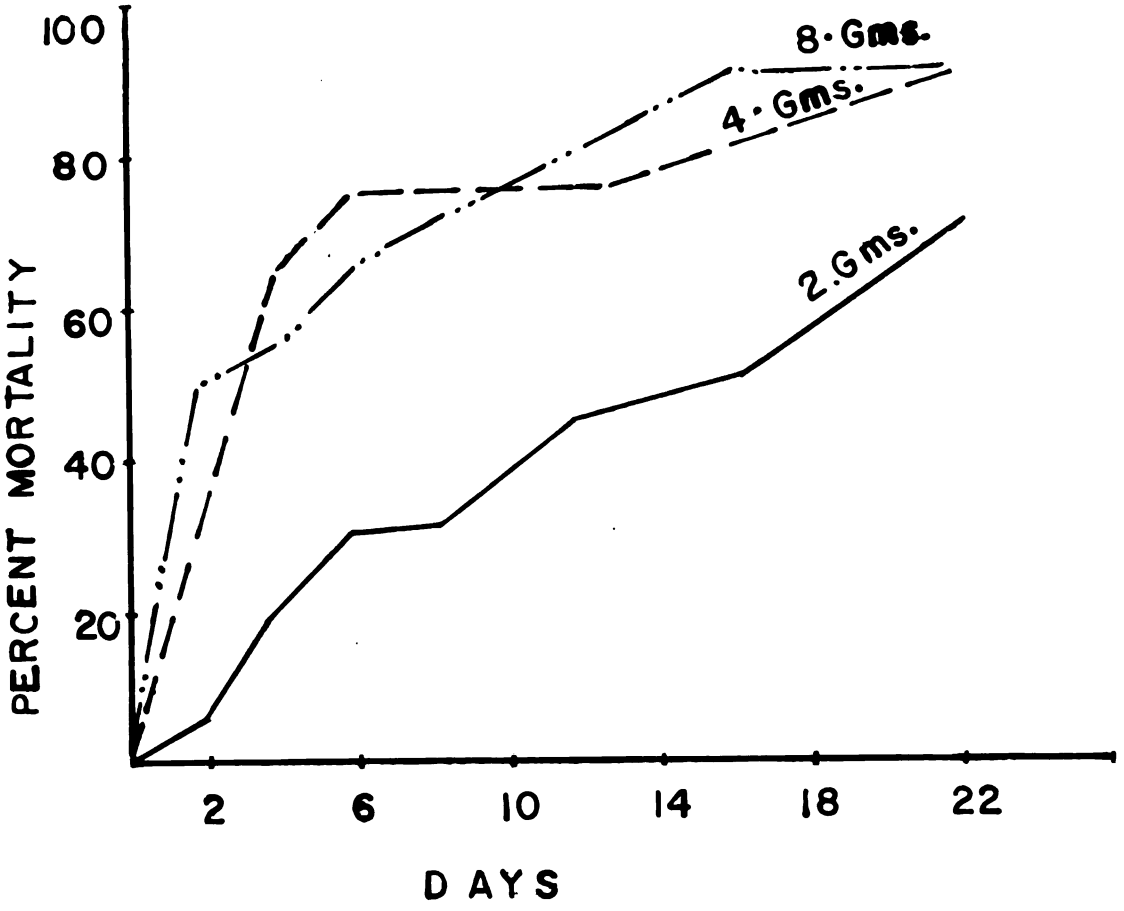

Fig. 5.--Thirteen-day-old larvae fed on various dosages of B. thuringiensis. (Test No. 2.)

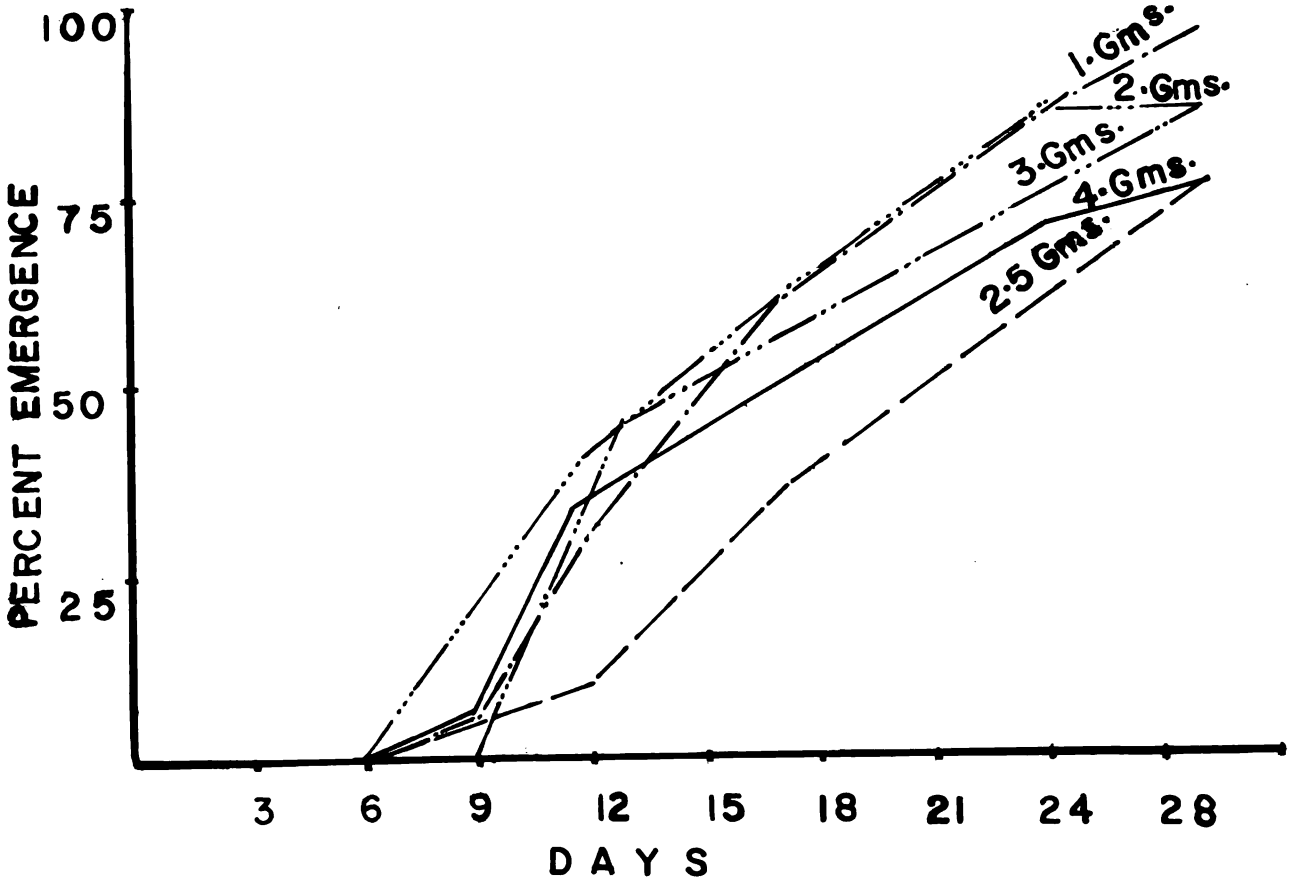

Fig. 6.--Percentage emergence from 16-day-old and over Galleria larvae fed on various dosages of B. thuringiensis.

MEASUREMENT OF RESPIRATION OF *GALLERIA* LARVAE TREATED WITH *BACILLUS THURINGIENSIS*

(a) Procedure

Metabolic rates of 7-day-old *Galleria* larvae were measured in terms of oxygen consumption using Warburg reaction vessels and standard manometric techniques (Umbreit *et al.*, 1957).

Two types of larval diets were prepared as explained earlier. One type was treated with *B. thuringiensis* spore preparation at the rate of 2 grams per 53 grams of diet and the other diet was untreated. The diets were kept for 24 hours in an incubator at 30^{o} C. after which a small amount of the diets was introduced into reaction flasks of the Warburg respirometer. Six reaction flasks were used for each diet type. Seven-day-old *Galleria* larvae of the same size taken from ordinary diet, were weighed and placed singly in reaction flasks. Twenty percent KOH was introduced to the center well of the reaction flasks to remove any carbon dioxide formed in the system. Two other reaction flasks were set up without larvae but one with treated diet and the other with untreated diet to act as diet respiratory controls.

The reaction flasks were attached to the manometers and their volumes were determined. Calibration of flasks and manometers under the conditions in which the flasks contained nutrient media and living *Galleria* larvae of unknown total volume required the use of a special gas calibration method (see Hoopingarner and Beck, 1960 for details). The experiment was run for one hour, changes in volume were recorded at

intervals of 20 minutes. The experiment was stopped and resumed again after predetermined time intervals. The experiment was run for 42 hours, at the end of which time the larvae were taken from the reaction flasks and weighed.

(b) Results

The results were expressed as average oxygen uptake in µl. per hour per average live weight of larvae.

TABLE 2.--Average oxygen uptake of 7-day-old Galleria larvae treated with 2 grams of B. thuringiensis per 53 grams of diet

	Treated		Check	
Time (Hours)	Average total weight of larvae (mg)	O_2 uptake per mg. live weight per hour (QO_2)	Average total weight of larvae (mg)	O_2 uptake per mg. live weight per hour (QO_2)
1	9.98	3.46 µl.	9.01	9.04 µl.
16:15	9.68	4.80 µl.	10.40	6.84 µl.
24:05	9.64	3.53 µl.	11.20	7.57 µl.
41:30	9.47	3.17 µl.	12.88	7.16 µl.

Considerable individual variations in the oxygen output for both treated and untreated larvae were observed. The treated larvae, however, showed a marked average decline in oxygen uptake per unit time (one hour) compared with the untreated larvae. The average pattern of this oxygen uptake as shown in Figure 7 is interesting. In the treated larvae there was a sharp rise in oxygen uptake followed by a sudden drop which then maintained a gentle slow rate. The untreated larvae on the other hand showed a sudden drop in oxygen uptake followed by a sudden rise.

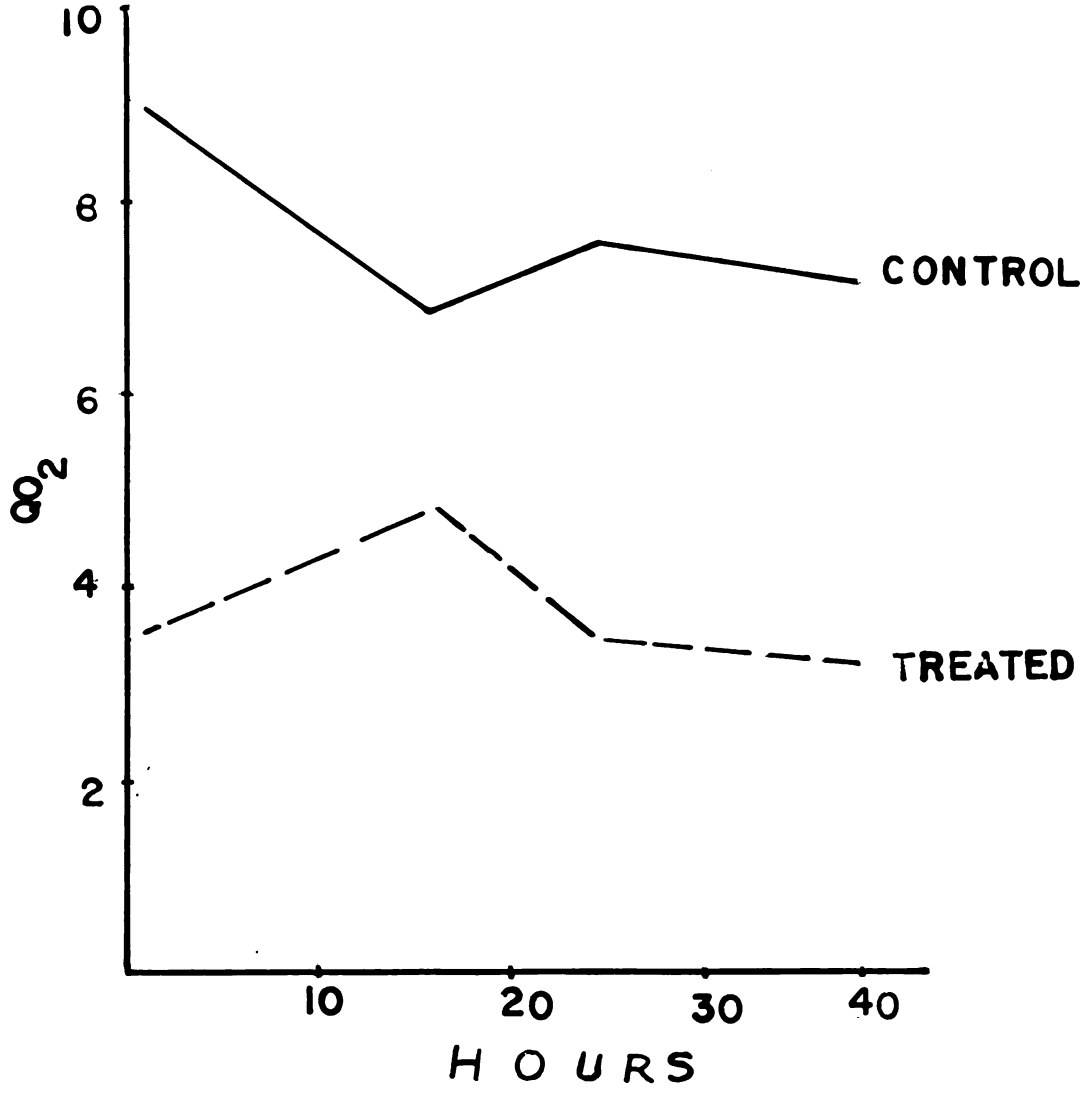

Fig. 7.--Average oxygen uptake of 7-day-old Galleria larvae treated with 2 gms. of B. thuringiensis in 53 grams of diet.

The comparative rates of oxygen uptake on μl./mg. live weight basis showed a similar pattern (Fig. 7). Table 2 also shows that the average weight of the treated larvae decreased while that of untreated larvae increased, though at a slower rate than that observed by Beck (1960).

TOXICOLOGICAL TESTS ON MEDITERRANEAN FLOUR MOTH, ANAGASTA (= EPHESTIA) KÜHNIELLA (ZELL.) WITH BACILLUS THURINGIENSIS

(a) Procedure

Mediterranean flour moth larvae were obtained from a stock reared in the laboratory. These were fed on a mixture of wheat flour (95%) and yeast (5%). Since the eggs are laid in the diet, larvae of the same age or size were not obtained. Therefore, in this experiment larvae of different sizes and ages were used.

The experimental diet was made by mixing B. thuringiensis spore powder with the diet to make 5%, 10%, 25%, 30% and 40% mixtures. Ten grams of each mixture was put on a petri dish to which 10 larvae were introduced, covered with a glass cover and put in an incubator at 25^{o} C. The same number of larvae were put on untreated diet in the same number of petri dishes to act as controls. Due to the limited number of larvae available, only one replicate of each was run.

The first counts of dead larvae were made at intervals of 3 days but later at longer intervals.

(b) Results

The experiment was run for 42 days and at the end of this period all larvae had either died or emerged as adults.

The results are summarized in Figure 8. The test showed that Mediterranean flour moth larvae are less susceptible than Galleria larvae to B. thuringiensis. By the 12th day only 20%, 30% and 40%

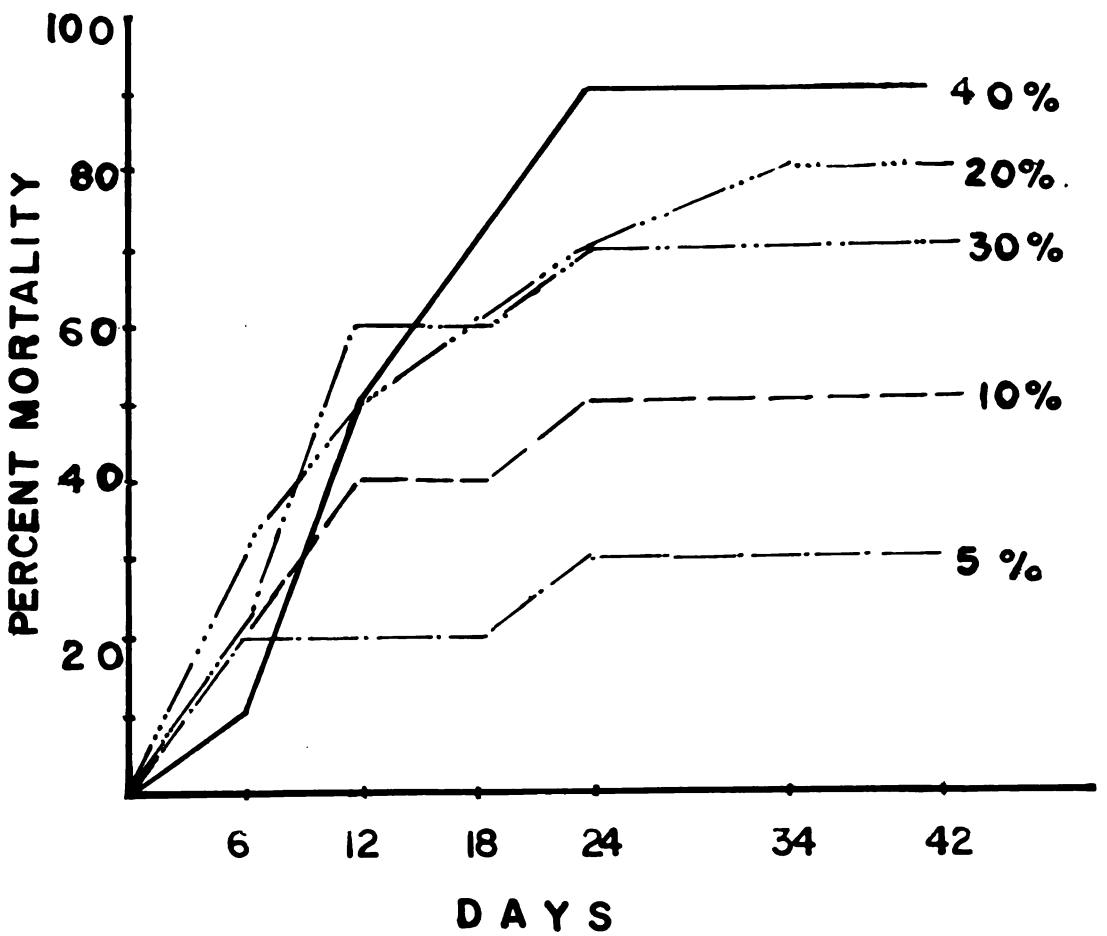

Fig. 8.--Percentage mortality of Mediterranean flour moth fed various dosages of B. thuringiensis.

bacillus spore formulations had effected 50% kill. The variations in the ages of the test larvae could have affected the results.

HISTOPATHOLOGICAL STUDIES ON GALLERIA LARVAE TREATED WITH BACILLUS THURINGIENSIS

(a) Procedure

Seven-day-old Galleria larvae feeding on normal diet were transferred to a diet treated with 2 grams of bacillus spore formulation per 53 grams of diet and put into an incubator at 30° C. After about 48 hours the larvae were removed from the diet, fixed in KAAD, infiltrated by a dioxane method (Guyer, 1953) and transverse sections made from them. Untreated larvae were similarly sectioned. Thirteen-day-old larvae were similarly treated and sectioned except that samples were taken at intervals of 4, 8, 12, 22 and 45 hours from the beginning of feeding.

The sections were cut 8-10 μ thick and stained either in iron haematoxylin and eosin or in Feulgen solution and fast green. Some mature untreated larvae were also sectioned.

The sections were examined microscopically and the structure of mid-guts of treated larvae were compared with those of untreated larvae.

(b) Results

The structure of the mid-gut epithelium in untreated larvae is a bit different for each age group but shows some agreement with the accounts of Tchang (1929). The large epithelial cells, the globlet cells and regenerative cells were seen. In treated larvae the arrangement of these different cells is less obvious and in larvae treated for 12 hours or more the whole epithelium showed signs of complete

disintegration. The arrangement of various cells was obliterated and many cells were floating in the gut lumen (see plates).

DISCUSSION OF RESULTS

As observed by Splittstoesser and McEwen (1961), there are sets of variables in insect bioassay which can affect the results. Some of these are associated with non-uniformity of test insects. In the toxicological tests on Galleria and Anagasta there was evidence of this variability since the test insects showed variability even when they were reared under optimum conditions.

These toxicological tests however, showed some agreements with those reported by other investigators on other insects. They found that heavy mortalities of larvae treated with B. thuringiensis occurred between the 2nd and the 5th day. They also reported that the younger larvae were more susceptible than the older ones. Tanada (1956) for example, reported that using sprays of 0.5 and 1 gram of spore preparation per gallon of water on the cabbage worm, Pieris rapae (L.), reduced the insect population in the field appreciably by the 2nd day and by the 6th day all the test insects were dead. With 0.5 grams of spore preparation per gallon of water most of larvae of the diamondback moth, Plutella maculipenis (Curtis), treated in the laboratory were dead by the 4th day. Tanada also experimented with the cabbage looper larvae, Trichoplusia ni (Hbn.), both in the laboratory and in the field. In the laboratory he found that though there were some larvae which died from infection some were resistant to B. thuringiensis, especially those collected in the field. The field test confirmed the laboratory results that the cabbage looper was partially resistant to B. thuringiensis

though he suggested that successful control could be effected with higher dosages when there is a moderate infestation.

The present study showed a situation almost similar to that found in previous studies. The younger larvae were more susceptible than the older ones and the heaviest mortalities occurred between the 2nd and 4th day. It was also found that after the initial high kill, some of those surviving go on living for some days though they do not increase in size. In an attempt to find out whether there was a correlation between high concentration and mortality, 13-day-old larvae were treated with 2, 4 and 8 grams of bacillus spore per 53 grams of diet. Irregularity in the larval mortality, as observed in the previous tests was evident (see Fig. 5). At the end of 22 days there was no difference in mortality for 4 and 8 grams of spores. Such a situation was reported by Smirnoff and Heimpel (1961) who found that, for earthworms, at 23^{o} C. there was no difference in mortality for 30 and 60 grams of spores in 300 grams of soil. At the end of the test (22 days) there were some live Galleria larvae even in the highest spore concentration, though they had hardly grown during the period they were on the treated diet. Those in the controls were all moths at the end of this period.

The mortality curves of 7-day-old larvae showed a definite plateau. For the 13-day-old larvae this plateau was less obvious but in the 13-day-old larvae treated with 2, 4 and 8 grams of spore per 53 grams of diet, there was a plateau of 6 days period though this was not as obvious at 22 days (Fig. 5). This tendency is probably what was called partial resistance by Tanada (1956). Figure 2 drawn with a log-dosage-probit transformation for 7-day-old larvae still shows this

plateau. That of 13-day-old larvae is not obvious.

Though inadequate tests were done with Mediterranean flour moth the results were in agreement with those of Jacobs (1950) who hinted at a partial success of B. thuringiensis to control this insect. The mortality curves again show the plateau pattern observed earlier with the highest kill within the first 12 days.

It is known that the structure of the mid-gut of most insect larvae undergoes many changes during the normal larval life (c.f., Tchang, 1929). Due to limited time those changes could not be traced in both treated and untreated larvae of Galleria. Some investigators (c.f. Mattes, 1927) have tried to systematize the course of action of B. thuringiensis in insects. They mention a stage of sporulation in the insect's body. In this study no spores were seen and as far as is known spores have not been observed in infected insects. They have however been seen in sections made from a dead infected earthworm (Smirnoff and Heimpel, 1961). More research is needed in this line to determine whether or not death is caused by toxins produced by the bacillus spores or by disintegration and/or blockage of the alimentary canal.

SUMMARY

Galleria mellonella larvae were found to be susceptible to *Bacillus thuringiensis*. Younger larvae were more susceptible than older larvae. Variations in bacillus activity were noted. A possible case of partial resistance was indicated though not confirmed. The infected larvae which survived showed marked retardation in growth and lowered oxygen consumption. Disintegration of mid-gut epithelium of infected larvae was observed but no spores were seen. More research is needed on the mode of action of *B. thuringiensis*.

A. kühniella larvae though susceptible to *B. thuringiensis*, were less so than *G. mellonella*.

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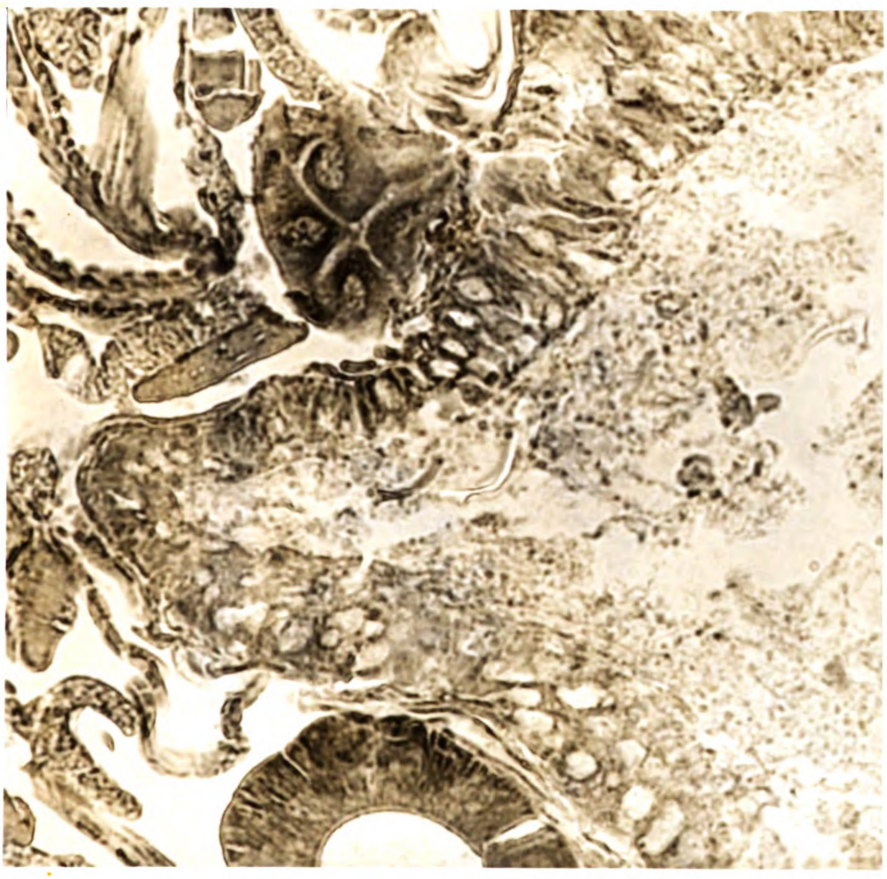
APPENDIX

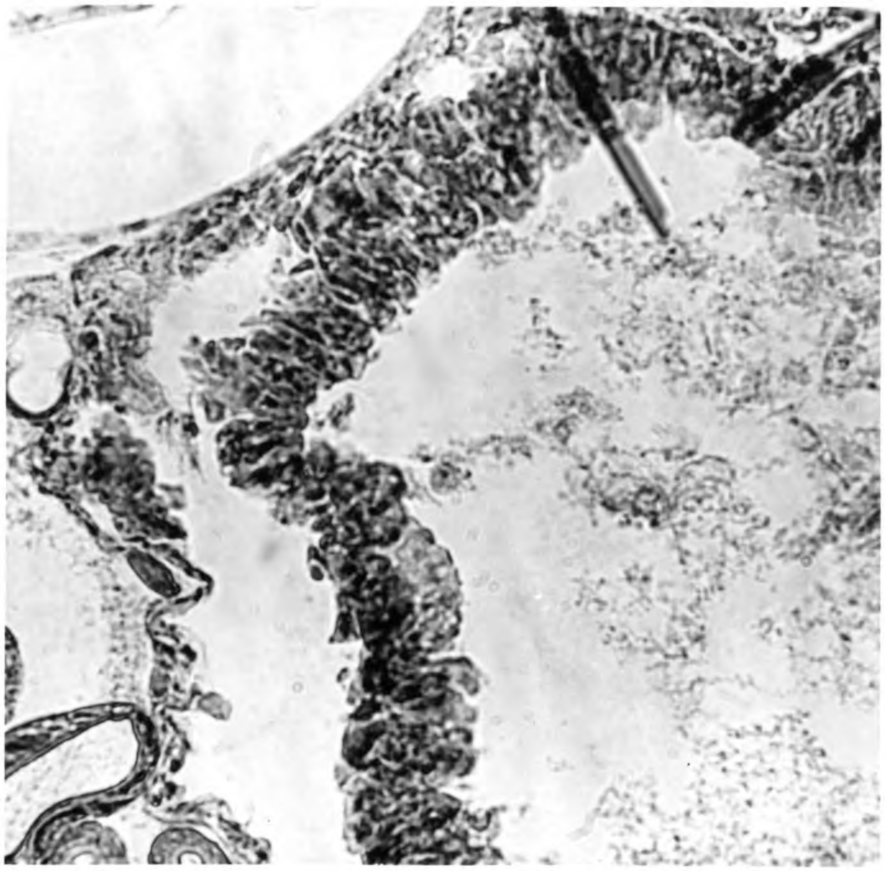
PLATE I



TRANSVERSE SECTION THROUGH THE MID-GUT OF A 7-DAY-OLD UNTREATED LARVA OF GALLERIA MELLONELLA (L.)

Fixed in Kahle's solution and stained in Iron haematoxylin-eosin. 10u (400X).
Epithelial cells are compact and form a continuous layer.

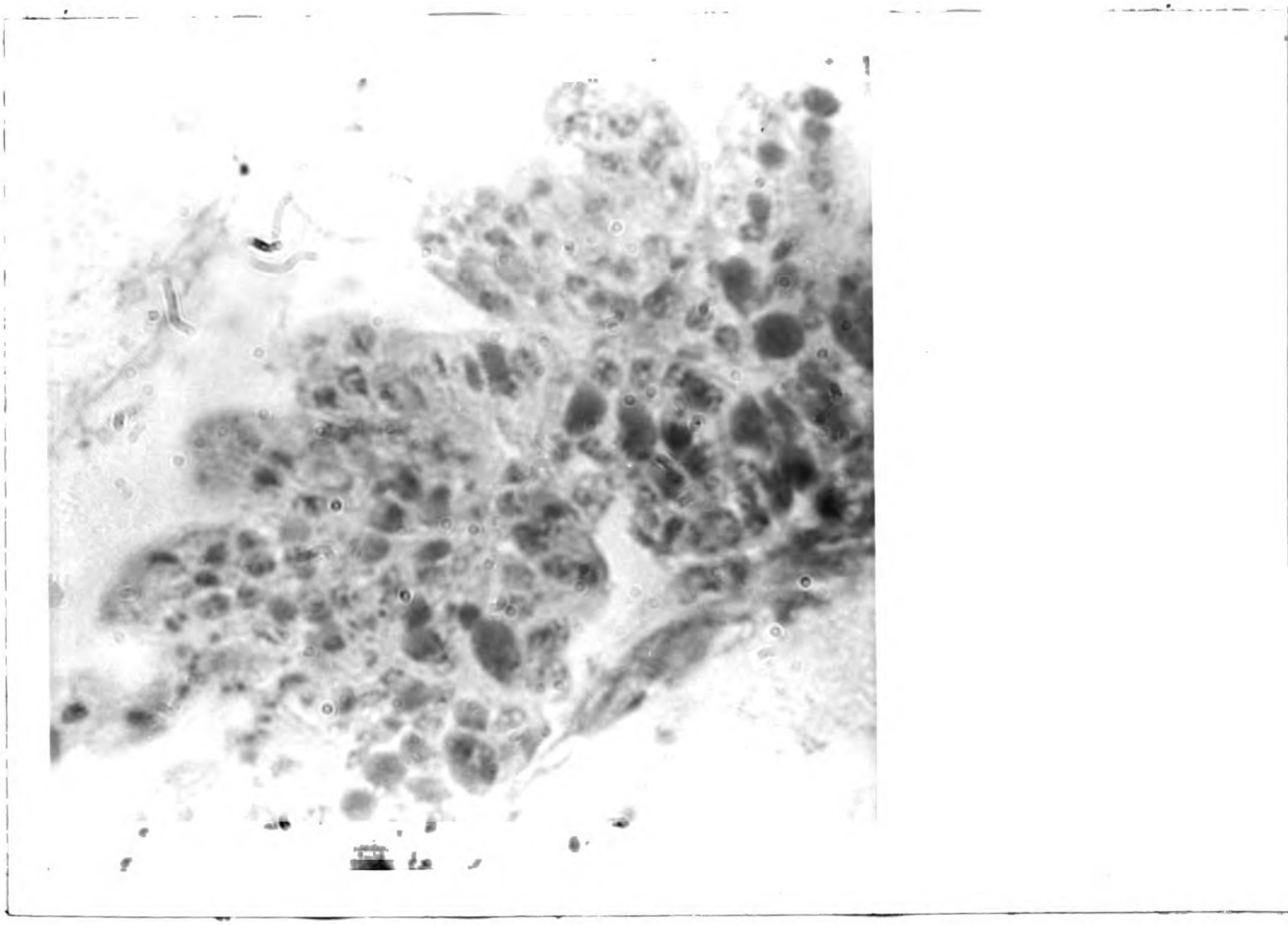
PLATE II



TRANSVERSE SECTION THROUGH THE MID-GUT OF A 7-DAY-OLD LARVA OF GALLERIA MELLONELLA (L.) FED ON DIET TREATED WITH 4% BACILLUS THURINGIENSIS BERLINER

Fixed in Kahle's solution and stained in Iron haematoxylin-eosin. 10u (400X).
Epithelial layer has disintegrated and cell masses are found in the mid-gut lumen.

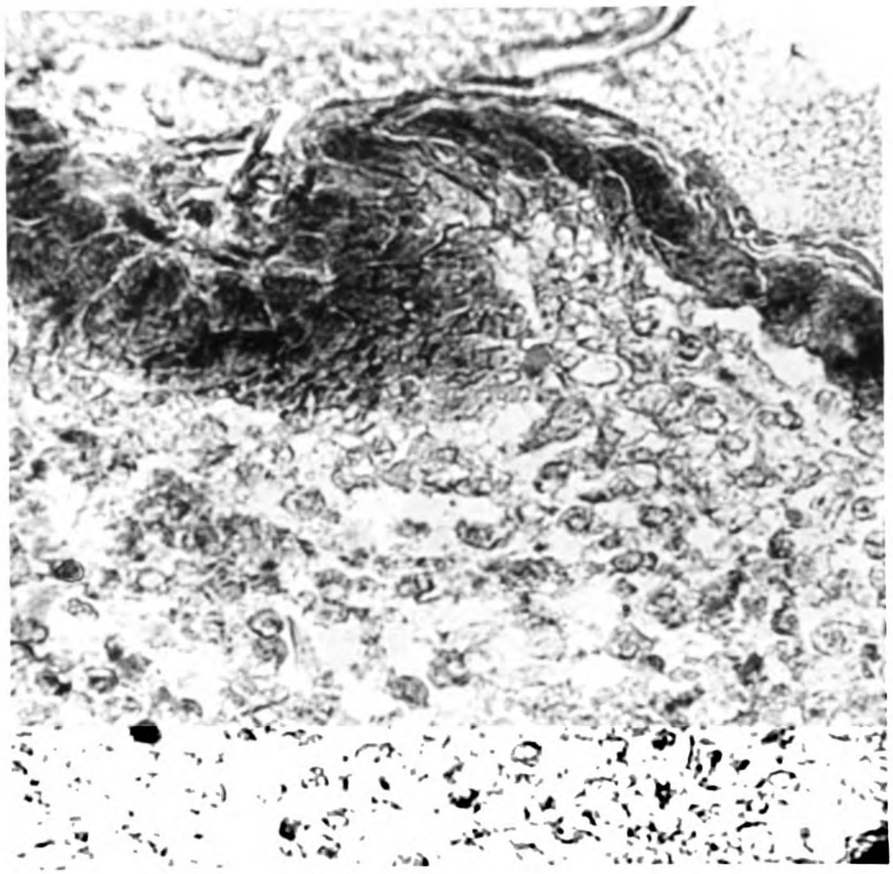
PLATE III



TRANSVERSE SECTION THROUGH THE MID-GUT OF A 13-DAY-OLD HEALTHY LARVA OF GALLERIA MELLONELLA (L.)

Fixed in KAAD and stained in Feulgen-fast green.
10u (850X).
Epithelial layer is compact and continuous with cells attached on the basement membrane.

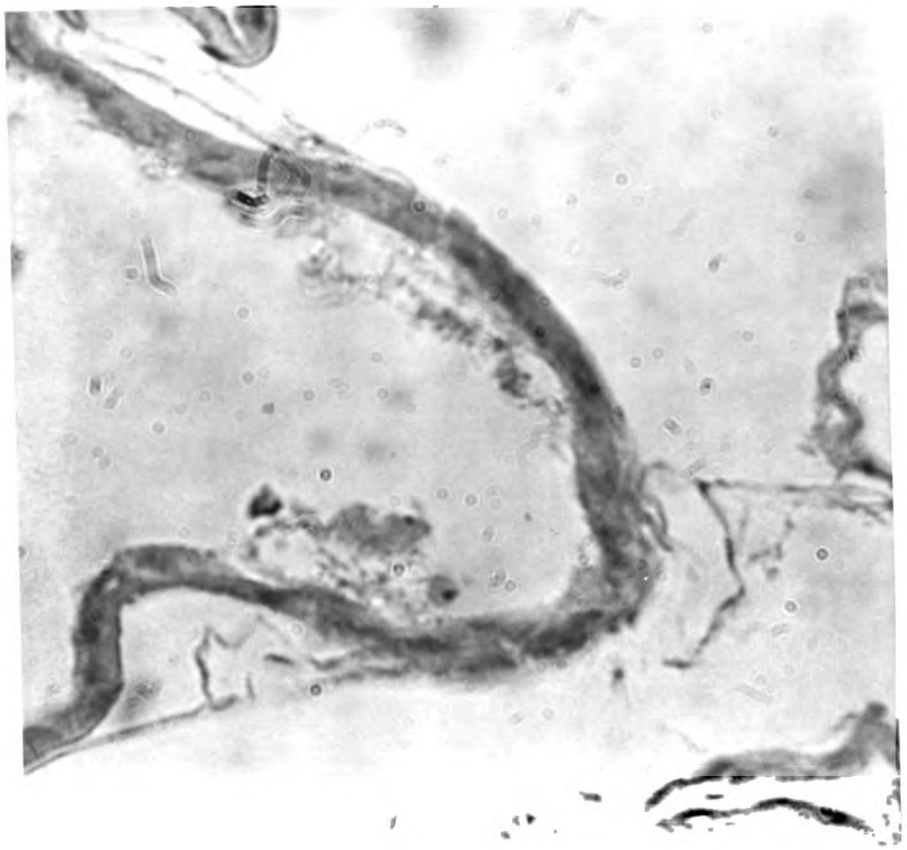
PLATE IV



TRANSVERSE SECTION THROUGH THE MID-GUT OF A 13-DAY-OLD LARVA OF GALLERIA MELLONELLA (L.) 45 HOURS AFTER FEEDING ON A DIET TREATED WITH 4% BACILLUS THURINGIENSIS

Fixed in Kahle's solution and stained in Iron haematoxylin-eosin. 10u (400X)
Epithelial layer has almost fallen into pieces and no longer continuous. Individual cells can be seen floating in the mid-gut lumen.

PLATE V



TRANSVERSE SECTION THROUGH THE MID-GUT OF A 13-DAY-OLD LARVA OF GALLERIA MELLONELLA (L.) FED ON A DIET TREATED WITH 4% BACILLUS THURINGIENSIS FOR 22 DAYS

Fixed in KAAD and stained in Feulgen-fast green.
10u (850X)
The epithelial layer has been reduced to a single cuboidal cell layer.