RELATION OF PROTEOLYTIC ENZYMES TO PHASE OF LIFE CYCLE OF *BACILLUS LARVAE*, AND TWO NEW CULTURE MEDIA FOR THIS ORGANISM

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INTRODUCTION

In the several descriptions of *Bacillus larvae* (White), the cause of American foulbrood of bees, that have appeared in the literature, there are discrepant findings concerning the ability of the organism to produce proteolytic enzymes. The work here reported, a continuation of a study made earlier by Sturtevant (1924), reveals that the elaboration of certain proteolytic enzymes by the bacillus is intimately related to its life cycle. This fact may help to explain some of the apparently divergent earlier results.

REVIEW OF LITERATURE

The gelatin-liquefying property of *Bacillus larvae* was not determined by White (1906), since, as he says, the organism fails to grow at the temperature at which gelatin remains solid. Maassen (1908), working at the same time, reported that a slow but gradual liquefaction took place unless inhibited by the presence of glucose in the medium. Sturtevant (1924) showed that protein decomposition as a result of the growth of *B. larvae* could be demonstrated, both in infected bee larvae and in egg-yolk broth, by chemical analyses, but direct action of the organism upon gelatin was not sufficient to be called definitely positive,

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although infected ropy larval material did produce rapid gelatin liquefaction. Lochhead (1928) found gelatin liquefaction to be variable, depending upon the strain of the organism used. In litmus milk he observed the formation of a rennet-type curd, without any peptonization, even after prolonged incubation. Stoilowa (1938) reported that her cultures of *B. larvae* liquefied gelatin and coagulated milk without hydrolysis.

Since *Bacillus larvae* fails to grow on the ordinary laboratory media, complex media have been devised for its cultivation, some of which are both difficult and tedious to prepare, as well as requiring materials not generally available. White (1906) first succeeded in growing *B. larvae* on a honeybee-larvae-infusion-agar. He later used sterile unheated brood filtrate in place of infusion (1907), and eventually he found that sterile unheated egg yolk added to the base medium (1919) could be substituted for the larval material. Sturtevant (1924) devised a medium in which sterile egg yolk was added to a yeast-peptone base. Later he added carrot extract (1930) to this medium and was able to obtain growth with a minimum inoculum of 50,000 spores per milliliter (1932). Lochhead (1928) found that when carrot extract is present in the medium an apparently specific nitrite test for *B. larvae* can be obtained. More recently Tarr (1937a, 1937b, 1938), working at Rothamsted, has utilized modifications of the larval-filtrate media, as well as a minced egg-embryo medium. The latter substrate enabled him to obtain growth, usually with inocula containing as few as 1,000 spores, and occasionally with approximately 140 or 100 spores. He used a larval-filtrate medium for the production of a spore crop with vegetative cultures. Stoilowa (1938) reported good growth on a glucose-blood agar.

EXPERIMENTS WITH DISEASED AND HEALTHY HONEYBEE LARVAE

When litmus milk was tested for suitability as a medium for the cultivation of *Bacillus larvae* from "scales,"² a surprising

² A diseased dead honeybee larva which dries down and adheres to the base of the brood cell is known to beekeepers as a scale. Each scale carries on an average approximately 2,500,000,000 spores.

reaction was noted; peptonization became apparent after only a few hours of incubation at 37°C., and was usually complete within 12 to 18 hours. Since vegetative cultures of *B. larvae* inoculated into milk produce only an acid reaction with reduction and curdling, it appeared that the proteolytic enzyme must have been contained in the scale prior to inoculation and be independent of any germination and growth following inoculation.

That this was indeed true was shown in several ways. First, a sterile, unheated filtrate of a scale suspension in water produced both peptonization of litmus milk and hydrolysis of gelatin; a similar reaction could be obtained with unheated and unfiltered suspensions in the presence of chloroform. Again, the same suspension peptonized milk and liquefied gelatin at 50°C., which is above the maximum temperature for the growth of *B. larvae*. Finally, the suspension was inactivated by heating to 93°C. for 20 minutes, although the spores subsequently germinated and reproduced.

To determine whether enzymes were produced by healthy honeybee larvae, the bacillus, or an interaction between the two, a series of healthy and diseased larvae were inoculated into litmus milk. It was found that unsealed larvae, which at this stage are constantly fed honey and pollen by the nurse bees, contained some proteolytic enzymes, possibly derived from the pollen content of the food. After the larvae were sealed over in the cells, being in the prepupal stage and no longer feeding, these enzymes disappeared in healthy individuals. On the contrary, diseased larvae, after being sealed, showed a much higher enzyme content as the symptoms of the disease increased. Also, as the severity of the disease increased, as indicated by decomposition, the percentage of spores compared with vegetative cells increased.

DEVELOPMENT OF NEW CULTURE MEDIA FOR BACILLUS LARVAE

For the study of pure cultures of *Bacillus larvae* and for use in the study of the production of enzymes on a solid substrate, two relatively simple media recently have been devised in the Intermountain States Bee Culture Field Laboratory at Laramie, Wyo., one for diagnostic purposes and the other for stock culture studies.

These media have the following advantages over those previously used: (a) Growth is produced with an inoculum of theoretically only one spore, as calculated from the dilution of a standard suspension; (b) it is readily prepared without special technique or materials; and (c) it is clear and transparent. The composition of the diagnostic medium is as follows:

Glucose.....	10 gram
Bacto neopeptone.....	10 gram
Bacto yeast extract.....	10 gram
Agar.....	15 gram
Carrot extract.....	200 ml.
Cysteine.....	80 mgm.
Distilled water.....	800 ml.

(Adjust to pH 6.8-7.0)

The nitrite test for *Bacillus larvae*, which, together with the microscopic appearance of the culture, appears to be highly reliable (Lochhead (1928), Sturtevant (1932), Hitchcock (1936)), also is obtained with this medium after a few days' growth. The lag phase before growth appears is markedly reduced compared with the egg-yolk medium, and sensitivity is greater. In fact, with one strain of *B. larvae* no growth was initiated with an inoculum of less than 50 million spores per milliliter on the egg-yolk agar, even after 30 days' incubation, whereas the new medium produced growth after 4 days with an inoculum of one or only a few spores.

The method of inoculation was that previously used by Sturtevant (1932). By this method the spores are suspended in sterile distilled water, and the number of spores per milliliter is determined by direct count under the microscope. From this standard suspension appropriate dilutions are made to obtain the desired number of spores per milliliter for inoculation of media. One milliliter of the dilution to be cultured is added to the slanted medium in a tube, which is then incubated at 37°C. in an upright position. It is to be pointed out that growths on the new medium with such small inocula are obtained only when the cells are added to the slant, in this case suspended in 1 ml. of sterile water, and the same results are not obtained even when much larger numbers of spores are added by the loop-inoculation method. In the

diseased larvae available for diagnosis and culture, the organism is found, almost invariably, only in the spore stage and generally in pure culture; hence the importance of obtaining spore germination as well as vegetative growth.

While the method described above is useful in routine diagnosis, it has a serious disadvantage as a stock medium. Although a large amount of vegetative growth occurs, few or no spores are formed, and the culture often dies out after a few weeks, the vegetative rods disintegrating. In a series of experiments it was found that whenever carrot extract was present in the medium sporulation was suppressed or inhibited, but when the carrot extract was omitted and neopeptone was present sporulation freely occurred. These data were obtained on vigorous strains of *B. larvae* capable of sporulation.

When the concentrations of the ingredients of the medium, without the carrot extract, were varied in order to approximate the optimum concentrations for growth and sporulation, it was found that the percentage of sporulation was highest in the medium made up to full strength, and such medium also gave the greatest total growth and consequently the greatest total spore harvest. This is contrary to the findings of Tarr (1937a).

The medium finally adopted for stock culturing, as well as for the production of spores for experimental purposes, is given below:

Glucose.....	10 gram
Bacto neopeptone.....	10 gram
Bacto yeast extract.....	10 gram
Cysteine.....	80 mgm.
Agar.....	15 gram
Distilled water.....	1000 ml.
(Adjust to pH 6.8-7.0)	

With this medium there is a slightly longer lag phase than with the carrot-extract type, and the nitrite test cannot be obtained on it, but it has been found very satisfactory for its particular use.

EXPERIMENTS WITH SPORES OF BACILLUS LARVAE ON NEW CULTURE MEDIUM

In order to ascertain whether a correlation similar to that described in diseased larvae existed between enzyme content and

phase of life cycle of *Bacillus larvae*, *in vitro*, to each tube of a series of slant cultures of various ages growing on the new stock medium 10 ml. of sterile litmus milk was added, and the whole incubated at 37°C. The results exactly paralleled those obtained with diseased larvae; that is, with young cultures not yet sporulating no peptonization occurred, but as sporulation began some peptonization could be noted, and older cultures which had completely sporulated gave a strong reaction after a few hours' incubation. Analogous results were obtained with respect to gelatin liquefaction. From this it would seem that these enzymes are released as the old cells disintegrate to free the newly formed spores. However, a parallel experiment with several apparently asporogenous strains of *B. larvae*, derived from sectors of giant colonies, showed that mere autolysis of vegetative cells without attendant sporulation engenders the production of no such enzymes. The appearance of these enzymes appears to be peculiar to the sporulation process.

It also appeared possible that these proteolytic enzymes were produced by the activity of the so-called dormant spores. Such activity by dormant spores has been claimed by Effront (1917), Reuhle (1923), Cook (1931), and Tarr (1934). The theory of Effront, that under unfavorable conditions dormant spores elaborate extra-cellular enzymes and are "the more productive of enzymes the more difficult their germination," was first examined.

A spore suspension was prepared, and a portion removed before heating to serve as a control. The remainder was heated in flowing steam for 15 minutes at 93.5°C., which inactivates the enzymes but does not notably affect spore viability. One-milliliter amounts of the control and of the heated suspension were placed in 5 ml. of sterile litmus milk and incubated at 37°C. Three 5-ml. amounts of heated suspension were incubated at 37°C. for 1 week, one without any further treatment, one with a small amount of toluene, and the third with a small amount of chloroform, and then tested by inoculation into litmus milk and incubation at 37°C. The results of this experiment, which was repeated with the same results, are given in table 1.

Here there are no indications of enzymatic activity by dormant

spores, nor did the presence of antiseptics induce such activity. Had such antiseptics stimulated enzyme elaboration, it seems reasonable that this would have been detected, inasmuch as the unheated control kept 1 week over chloroform did not lose its potency.

The possibility that the heating had destroyed the capacity of the bacterial protoplasm to form enzymes was examined. The spores from a heat-inactivated suspension were cultured on the stock medium, and, after growth and sporulation had occurred, a suspension of these spores proved to be active again in hydrolyzing litmus milk. Such heating, then, seemed to have no permanent effect on the culture.

TABLE 1
Peptonization of spore suspensions in litmus milk

TREATMENT OF SUSPENSION	PEPTONIZATION AFTER	
	8 hours	20 hours
	<i>per cent</i>	<i>per cent</i>
Unheated.....	50	100
Heated.....	0	0
Heated and held 1 week.....	0	0
Heated, toluene added, and held 1 week.....	0	0
Heated, chloroform added, and held 1 week....	0	0
Unheated, chloroform added, and held 1 week..	50	100

Reuhle (1923), basing his conclusions on data obtained with twice-washed bacterial spores, stated that the spores secreted enzymes even though they did not germinate. Experiments were set up to determine whether such a mechanism was operative in the writers' own case. It was found that suspensions of *Bacillus larvae* spores could be centrifuged and washed three times without being completely freed from proteolytic enzymes, as tested by inoculation into litmus milk. Further washings, though, yielded suspensions showing no casein hydrolysis or gelatin liquefaction, even after prolonged incubation.

CONCLUSIONS

The above data indicate that the proteolytic enzymes found in honeybee larvae infected with *Bacillus larvae*, or in scales of

larvae dead from such infection, are not elaborated by the honey-bee larval organism. Larvae in the pre-sealed stage, at which time they are consuming pollen and honey, do contain enzymes which liquefy gelatin and peptonize milk, but in healthy larvae, after being sealed and in the prepupal stage, the enzymes can no longer be demonstrated. That these enzymes are released by the bacterial organism as it sporulates is evidenced by the fact that the amount of enzyme in diseased larvae is in direct proportion to the percentage of spores, and although in vegetative cultures of *B. larvae* no such proteolytic enzymes are found, they appear concomitantly with sporulation, increasing with further sporulation. Production of these enzymes by dormant spores could not be demonstrated.

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