INTRACELLULAR BACTERIOIDS IN THE COCKROACH
(PERIPLANETA AMERICANA LINN.)

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Problems involving the so-called "intracellular symbionts" have long troubled some cytologists and bacteriologically inclined zoologists. The solution of many of these problems, however, yet evade even the most careful investigators.

Several workers in this field (Buchner, Glaser, Mercier, Schwartz) hold to the view that the physiology and systematic position of the intracellular bacteroids can be accurately determined only by the cultivation of these organisms on artificial media. Toward this end, many workers (see Schwartz, 1935) have struggled with sterilization and dissection techniques and experimented with various culture media in what has usually been futile or unsatisfactory attempts to grow the bacteroids in unnatural conditions.

The extracellular "symbionts" in the gut of insects are generally not strict in their growth requirements (Schwartz, 1935; Steinhauis, 1941), and some of the forms that have an intracellular stage in the gut wall and an extracellular stage in the gut lumen seem to grow readily on artificial media from their extracellular stage: e.g., those of species of Sitodrepa, Ernobius, Rhaqium, Xestobium (Heitz, 1927; Muller, 1934), Rhodnius (Wigglesworth, 1936), and others (see Schwartz, 1935, p. 398). The more closely adapted intracellular organisms, however, grow with difficulty if at all, and none of the reports of successful cultures of these are above serious criticism on matters of sterilization and manipulation.

The intracellular bacteroids of the cockroaches, with which this paper deals specifically, have been so thoroughly described (Fraenkel, 1921; Gier, 1936; Bode, 1936; Hoover, 1945) that it suffices here to say that these bacteroids are nonmotile, nonsporeforming, faintly gram-positive, straight or slightly curved rods, 0.8–1 μ by 1.5–6.5 μ, and may stain barred, somewhat like diphtheroids. They occur around the ovarian eggs and in specialized cells, the mycetocytes of the abdominal fat bodies.

1 The work reported here was begun at Indiana University under the direction of Dr. Fernandus Payne, as part of a doctorate problem, and was continued at Harvard University Biological Laboratories on a National Research Council Fellowship and a Harvard University Research Fellowship under the general supervision of Dr. L. R. Cleveland and Dr. A. B. Dawson. Aid and counsel which made this work possible and profitable are gratefully acknowledged.

2 The term "symbiont" definitely connotes a helpful association between two types of organisms. Such a relationship has never been demonstrated for any of the true "intracellular symbionts." Mercier (1907) designated the bodies of the roach mycetocytes as "bacteroidi" and this lead was followed by Hertig (1921), Gier (1936), and Hoover (1945). It seems better thus openly to confess our ignorance as to the nature of these bodies by continuing to designate them merely as "bacterialike" than to imply a relationship which probably does not exist, even though these "bacteroidi" may sometime be definitely placed taxonomically with the bacteria.
There have been alternate reports of success and failure in attempts at culti-
vating these bacteroids since the early failures of Blochmann (1887), Kras-
siltschik (1889), and Forbes (1892). Mercier (1907), Glaser (1920, 1930),
Gropengiesser (1925), and Bode (1936) reported success, but Javelly (1914),
Hertig (1921), Wollman (1926), and Hovasse (1930) admitted failure. These
efforts have recently been summarized by Buchner (1930) and Steinhaus (1940).

Mercier (1907) cultivated, from the ootheca of *Blatta orientalis*, a motile,
sporoforming bacillus which he named *Bacillus cuenoti*. These cultures had the
effect of dispelling permanently the idea championed by Cuenot, Prenant, and
Heneguy that the “symbionts” of the roaches and other insects were only
metabolic products. Mercier’s work was discredited by the failure of Javelly
(1914) and Glaser (1920) to cultivate *Bacillus cuenoti*. Hertig (1921), in turn,
showed quite definitely that the spirillum cultivated by Glaser (1920) was not
the “symbiont.” Gropengiesser (1925) and Bode (1936), however, cultivated
motile, sporoforming rods from *Blatta orientalis* and *Periplaneta americana*,
respectively, which they concluded were identical with *B. cuenoti* in spite of
certain discrepancies in the published descriptions. Mercier (1907) and Gro-
pengiesser (1925) also frequently cultivated a yeast that they believed was a
secondary “symbiont” that could, on occasion, displace the bacteroids, but
neither gave any evidence for his contention. Glaser (1930), in a series of very
carefully executed experiments, cultivated three strains of diphtheroids from
*Periplaneta americana* and attempted to prove serologically that they were the
“symbionts.” More recently, Hoover (1945) has reported successful cul-
tivation of diphtheroids and other bacilli from *Cryptocercus*.

In view of these conflicting results, it seemed desirable to check critically the
various techniques and media used in past cultivation experiments, to try new
methods, and to attempt to analyze results more thoroughly.

The first difficulty, and the source of the most constant error in such cul-
tivation experiments, is the problem of securing the “symbiotic” organism from
the host tissue without contaminating the material with bacteria that may
subsequently be mistaken for the “symbiont.” The ideal way to eliminate
contaminants is to rear the insects aseptically from previously sterilized eggs.
Wollman (1926) and Bode (1936) developed techniques for doing this with
*Blatella germanica* and *Periplaneta americana*, respectively, but both failed in
culturing any bacteria from such sterile roaches. The most convenient method
of obtaining uncontaminated, “symbiont-laden” material is the sterilization of
the oothecae chemically, using the contents of the oothecae directly as inoculation
material. The third and most treacherous method is the chemical sterilization
of the exterior of the roach and the removal of the “symbiont-laden” tissues.
Glaser (1920, 1930), Hertig (1921), Gropengiesser (1925), and Bode (1936),
have developed fairly satisfactory techniques along these lines.

The second and possibly the greatest difficulty in such cultivation experi-
ments is the provision of adequate culture media for the “symbionts.” Since
the physiological and chemical properties of the natural habitat of these organ-
isms are incompletely known, an adequate medium can be found only by the
trial and error method. It would be expected that organisms as highly specialized as the intracellular "symbionts" would require a very special medium. Mercier (1907) and Gropengiesser (1925), however, cultivated Bacillus eunoti readily on most routine bacteriological media. Schwartz (1924) used a general medium with high sugar content for the yeastlike "symbionts" of the Lecanidae. Meyers (1925) used routine beef extract peptone media enriched with an extract of snails on which to cultivate the "symbionts" of the concretion organs of certain snails. Glaser (1930) depended on blood media for the cultivation of the roach symbionts, and Hoover (1945) followed Glaser's techniques. All these workers reported successful cultures with their respective methods, but their results have not been confirmed.

The third, and probably most perplexing, problem in "symbiont" cultivation lies in the identification of the cultivated organism. In the past, morphologic similarity between the cultivated form and the intracellular form plus dependence on the adequacy of the technique used have been the main criteria, and these, as will be shown later, are not reliable. Glaser (1930) attempted identification by serological comparisons, which to date has not been developed to reliability.

**Materials and Methods**

For the following series of experiments, the American cockroach, Periplaneta americana (Linnaeus), was used most extensively as the source of inoculation material, being supplemented at times with Blatta orientalis (Linnaeus), Periplaneta pennsylvanica (De Geer), and Cryptocercus punctulatus Scudder. All forms except the last were successfully reared in the laboratory (Gier, 1936, 1946).

Sterilization, dissection, and inoculation techniques were modified from those described by Hertig (1921) and Glaser (1930). Nymphs and adults to be used for bacteriological work were kept on clean filter paper in glass bowls, without food, for several days, so they would be as clean as possible and have little material in the gut. On removal from the bowl, each animal was pressed lightly to remove fecal pellets. Sterilization and dissection were conducted as follows: the roach was etherized until completely immobile, dipped into 95 per cent alcohol, agitated for 5 minutes in a solution consisting of equal parts of 1:500 mercuric chloride and 95 per cent alcohol, then rinsed in 70 per cent alcohol. The animal was then placed on its back in a dish of freshly melted and solidified paraffin, and secured with pins: one through the edge of the prothorax, one through the tip of the abdomen, and one on each side of the body posterior to the metathoracic legs crossing over the body and holding the legs forward well out of the way (figure 1). Then with a pair of fine scissors all the abdominal sternites, except the last, were cut along their left margins; the sternites were carefully grasped by their free margin with sterile forceps and the entire ventral body wall, as a unit, was turned over to the right and secured there with one or two pins. With fine forceps portions of the fat body or the ovary from the right side (side opposite the cut) were removed, separated from trachea and Malpighian
Materials for bacteriological examination were always taken from the side opposite the incision, thus precluding any possibility of external contamination. (Natural size)

The depression at the tip is of such a size that it holds the capsule firmly, yet without danger of crushing. (Natural size)

The stage shown as C is possibly the condition described by Mercier and Cuenot as yeast that had invaded the mycetomes and displaced the normal bacteroids. (X 2,000)

(Fig. 1) Photograph of a Mature Periplaneta americana, Showing Method of Securing the Roach for Removal of Tissues

(Fig. 2) Forceps Used for Holding the Ootheca for Removal of Embryos Aseptically

(Fig. 3) A Clump of Roach Bacteroids Showing Progressive Stages of Vacuolization in Hypotonic Solution

(Fig. 4) Section of Ootheca to Show Relationships of Embryos, Mucus Layer, and the Fused Edge of the Capsule. (X 2)
tubules in sterile Belar's solution, and transferred to the media in which they were macerated.

When eggs or embryos were to be used as the source of the inoculum, special precautions were taken to get the best possible oothecae. In order to keep a plentiful supply of embryos, vigorous adult females were kept in clean glass bowls (250-mm biological specimen dishes of the type that will stack) with plenty of food and water. The oothecae were taken from the females as soon as they were complete, which was usually 12 to 18 hours after their appearance from the vagina, and were stored in clean watch glasses. Sterilization and dissection techniques were the simplest possible. A perfectly formed, clean, unshriveled ootheca of the age desired was selected from the stock; it was dipped into 95 per cent alcohol, placed in the mercuric chloride, alcohol solution for 15 minutes, and rinsed in 70 per cent alcohol. One end of the ootheca was then grasped firmly with special forceps (figure 2) and the other end sliced off with a red-hot razor blade and discarded. The two eggs thus exposed were removed with a hot inoculating needle and discarded; the remaining eggs were transferred directly to the media, or they were macerated in the capsule with a sterile inoculating loop before the transfer was made.

All instruments were sterilized either in open flame or boiled in 70 per cent alcohol. The sterilizing fluids were freshly boiled and cooled. Sterile pins and other small instruments were all handled with sterile forceps. Dissections and inoculations were done in a tightly closed room that had been scrubbed the previous afternoon and sprayed well with dilute phenol solution shortly before the work was begun. As an added precaution against air contaminants, the last series of experiments was done under a sterile hood, and the bare arms of the worker were sterilized with mercuric chloride, alcohol mixture.

The efficiency of these methods needs little comment other than to point out that few contaminations occurred, as is shown in table 1. Absolute sterilization of either cockroach or ootheca is probably impossible because of rather frequent infections in the oviducts, Malpighian tubules, traches, or hemocoel. The time necessary for the application of the sterilizing fluids was determined, after much experimenting, as that sufficient to perfect external sterilization, and yet not enough to damage the animals seriously. Cockroaches that were allowed to revive after sterilization lived normally; and embryos in about 80 per cent of unopened, sterilized oothecae continued normal development.

The media used included Petroff's egg medium, Loeffler's coagulated blood serum (both horse and calf serum), deep brain media, potato, nutrient gelatin, beef extract broth with agar, Hutoon's hormone broth with agar, and modifications especially of the latter two. Beef extract and hormone broths were used as the basis for blood media (2 to 20 per cent defibrinated blood from horse, cow, or human added), and roach extract media (1 to 10 per cent extract from roaches added). This roach extract was made by either boiling, macerating, and filtering, or by digesting with trypsin, quantities of roaches, usually with alimentary canal removed, and was sterilized by the Berkefeld filter, inspissator, or autoclave. Inoculations were made both in liquid media and on solid media,
the latter being preferred because of better isolation of contaminants. Petri plates were inoculated by spreading macerated eggs, embryos, or fat bodies over the surface of the media in four or five consecutive spots, which were left separated by a few millimeters' space only (Glaser, 1930). This gave a dilution so great that rarely did any growth occur on the last two spots. In many cases a second plate was inoculated without reinfecting the loop, in order to check Glaser's (1930) theory that the symbionts will grow only if their natural inhibiting agents are greatly diluted.

The reaction of the media used was varied from pH 6.4 to pH 7.8 to cover the complete range of findings of hydrogen ion concentration in insect blood (Glaser, 1925; Bodine, 1926; and others), but most were adjusted to pH 7.2 because of my own findings on the hydrogen ion concentration in Periplaneta americana blood, on the assumption that the pH of protoplasm is the same as that of the surrounding blood.

The pH of the blood was determined colorimetrically and checked with a Gesel quinhydrone electrode. A drop of phenol red indicator was placed on an opal plate, one antennae of the roach clipped off, and the blood run directly into the indicator. Readings taken immediately were invariably 7.1 to 7.3, rising within 2 minutes to 7.4 to 7.5. For the electrometric check, blood was drawn into the Gesel chamber directly from the cut antennae and a reading taken as quickly as possible. The result was 7.3 ± 0.1 when one animal supplied sufficient blood, or 7.4 ± 0.1 if the blood of two animals was used. Tests made after 10 minutes or more invariably ranged between 7.55 and 7.65. The lower hydrogen ion concentration in the latter case is probably due to the loss of carbon dioxide and cannot be considered as normal. Samples of blood were diluted 1:16 with water without changing the pH more than 0.1 point, indicating a very efficient buffer action. Crushed cells in the test solution invariably increased the acidity to pH 5.8 to 6.8.

The salt content of the media was varied from 0 to 1 per cent, and the osmotic pressure was further increased at times by the addition of sugars, urea, sodium acid phosphate, potassium sulphate, and other salts in an attempt to make the media isotonic with the cockroach blood, which was found to depress the freezing point approximately 0.9 C as against 0.62 C for horse blood, which indicates a much higher concentration of salts in the roach blood.

All media were incubated 3 days at 30 C before they were inoculated, and all plates or tubes showing any contamination were discarded. About 200 cultures were tried under anaerobic conditions as stabs, shakes, tubes sealed with oil, Kumwiede-Pratt plates, and Novy jar cultures with the oxygen completely or partially displaced with carbon dioxide or the oxygen removed with pyrogallol. Most cultures were incubated at 30 C, as that was found to be the optimum temperature for Periplaneta americana (Gier, 1946). Others were incubated at room temperature or at 36 C. The inoculated media were examined daily for growths, and everything but obvious contamination, i.e., colonies between the inoculated spots, was carefully checked. Inoculated spots showing no growth after 3 or 4 days were carefully rubbed up with a drop of condensation fluid as
described by Glaser (1930), and material was transferred to slants of the same kind of media. These subcultures were examined, and the slant surface was flooded with condensation fluid daily for at least 10 days. If the agar became dry, a few drops of serum broth were added (Glaser, 1930). On the fourth or fifth day of incubation, whether or not a macroscopic growth could be seen, new transplants were made, and slides were prepared, stained, and examined microscopically from each original subculture.

RESULTS

Series I. This series of approximately 500 culture attempts was conducted at Indiana University. Materials for inoculation were taken in about equal numbers from Periplaneta americana, Blatta orientalis, and Parcoblatta pennsylvanica. The technique and media used were in general those described above, with emphasis on no one kind of medium. The results of this series were not at all convincing because of the high incidence of positive cultures on plates (about 35 per cent of all plates showed growths) and the great variety of organisms in these cultures. Most of these positive cultures were readily identified as contaminations by direct correlation of the cultivated organisms with bacteria occurring commonly in the environment of the roach. With each refinement of technique, however, the incidence of positive cultures declined so that, before work on this series was terminated, the sporeforming rods comparable to Bacillus cuenoti were of rare occurrence. Six cultures of diphtheroids, which were not readily explained as contaminations, were isolated in this series by subculturing apparently sterile spots. These were very similar to the diphtheroids described by Glaser (1930).

Series II. This series, conducted at Harvard University, was for the most part a duplication of series I except for greater refinements in technique and in the use of Periplaneta americana as the source of inoculum, supplemented with Cryptocercus punctulatus. Dissections were done on 15 days, at intervals of

<table>
<thead>
<tr>
<th>SOURCE OF INOCULUM</th>
<th>NO. OF TRIALS</th>
<th>PLATES CONTAMINATED</th>
<th>ORGANISMS PRESENT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heavily</td>
<td>Slightly</td>
</tr>
<tr>
<td>Oothecae</td>
<td>93</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Ovaries</td>
<td>39</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Fat bodies</td>
<td>69</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>201</strong></td>
<td><strong>18</strong></td>
<td><strong>13</strong></td>
</tr>
<tr>
<td>Water (controls)</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

TABLE 1

Attempts at cultivating the intracellular symbionts of Periplaneta americana and Cryptocercus punctulatus

(Series II; see text for explanation)
approximately 2 weeks. Twelve to 15 plates, plus controls, were inoculated on each dissection day.

The results are given in table 1. Of the 201 plates inoculated, 170 remained apparently sterile for at least 3 days. Growths on the 30 plates were, for convenience, designated as heavy contaminations, with a general heavy growth over any part of the plate, or as slight contaminations, with a few isolated colonies affecting only one or two spots. The organisms growing on these plates were of many different kinds, including a number of molds not listed in table 1, but all were found frequently as air contaminants on control plates or in cultures from the gut and from the exterior of the roach. Besides the contaminations listed, there were a total of 20 contaminating colonies on the plates definitely off the inoculated areas.

Of the 782 subcultures (table 2) from apparently sterile spots, only 28, or 3.58 per cent, showed any bacterial growth within the 10 days the cultures were kept under observation. These 28 positive cultures were of at least 12 different kinds of organisms: i.e., at least two kinds of yeasts, four kinds of diphtheroids, three kinds of other bacilli, two kinds of staphylococci, and one Sarcina.

Series III. A number of attempts to grow the "symbionts" in vivo was conducted after the failure of bacteriological cultures became evident. In one experiment 25 hen eggs were incubated at 37 C for 8 to 10 days. A small triangular window was cut through the egg shell, and a roach embryo or clump of fat body was implanted on the chorioallantois, with adequate precautions against contamination. The hole in the shell was sealed with paraffin, and the egg was returned to the incubator at 35 C. Temperatures higher than this were usually fatal for roach embryos, and hence would probably be unfavorable for the bacteroids. Sixteen of the chick embryos lived until the eggs were reopened 5 to 7 days later. In most cases the roach tissue was walled off and was in the process of being absorbed; in two, the inoculum was not located, and in three others, the roach embryo apparently provoked no reaction from the chick and both continued normal development to the end of the experiment. In another experiment, suspensions of bacteroid-bearing fat bodies were injected with a

### TABLE 2

Results from subculturing apparently sterile spots of plates listed in table 1

<table>
<thead>
<tr>
<th>ORIGINAL SOURCE OF INOCULUM</th>
<th>TRANSPLANTS</th>
<th>ORGANISMS ON TRANSPLANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Oothecae</td>
<td>384</td>
<td>10</td>
</tr>
<tr>
<td>Ovaries</td>
<td>144</td>
<td>1</td>
</tr>
<tr>
<td>Fat bodies</td>
<td>254</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>782</td>
<td>11</td>
</tr>
<tr>
<td>Water (controls)</td>
<td>41</td>
<td>0</td>
</tr>
</tbody>
</table>

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capillary pipette into the amniotic cavities of five 7-day-old chick embryos. On the fourteenth day, the eggs were reopened, but only degenerating bacteroids were found.

**Series IV.** Many attempts were made to grow the bacteroids in tissue culture, both in tubes and in hanging drops. Fat body clumps kept in drops of roach blood gradually lost their bacteroids during a period of about 2 weeks. The way in which the bacteroid number decreased was not definitely determined. Fat body clumps and bacteroid-laden portions of embryos lived in apparently normal condition for as long as 3 weeks in small tubes of media consisting of peptone meat extract broth 7 parts, horse blood serum 2 parts, and 10 per cent glucose solution 1 part, reaction adjusted to pH 7.0 to 7.2. In these cultures there was no indication of bacteroid growth, although about 1 out of 8 showed contaminating bacterial growth. Sixty hanging drop cultures of embryonic tissues, fat bodies, or gonads in roach blood, crayfish blood, cricket blood, horse serum broth, or chick amniotic fluid showed no signs of bacteroid growth although some of these were maintained, with two transplants, as long as 3 weeks.

Various and numerous controls were run concurrently with the inoculation experiments as follows:

**Air controls.** At the time of each series of dissections, a plate of the medium used for inoculation was left open on the table or under the hood for 30 minutes. A number of different kinds of bacteria found on the inoculated plates were found also in these air controls.

**Inoculation technique controls.** During the course of each series of inoculations, one plate was spotted in the regular fashion, using as inoculum sterile Belar’s solution such as was used for washing the roach tissues. In 10 such plates, only one colony, a gram-negative rod, appeared.

**Sterilization technique controls.** Frequently plates or tubes of media were inoculated with non-bacteroid-bearing parts of the roach body, as a leg, a segment of muscle, a portion of the ventral body wall, or a clump of Malpighian tubules. Positive cultures of various sorts occurred in approximately 1 out of 10 from muscle. Inoculations with large clumps of fat body showed nearly the same incidence of infection and the same bacteria as did inoculations with Malpighian tubules—20 per cent positive cultures. Oothecae placed in broth after they had been sterilized and the embryos removed produced positive growths, usually of yeasts and sarcinae, in about one case in four. The same organisms were obtained in the same relative frequency by inoculating with only the lips of the sterilized ootheca clipped off beyond the tips of the eggs, showing conclusively that most of the contaminations from oothecae come from between the lips where chemicals do not reach them and where they are not normally disturbed by the dissection technique used in this work; but the contamination would be transferred if the ootheca was opened along the seam or macerated. Numerous tubes of media (nutrient agar slats, broth, and gelatin stabs) were inoculated with bacteroid-laden and non-bacteroid-laden tissues. In these, positive cultures were relatively less frequent than with plates because chance for air contaminants was reduced to a minimum, but the same contaminants were encoun-
tered. Broth cultures were extremely unsatisfactory because of the uncertainty of the original quantity of contaminant.

A considerable number of sterilized oothecae were placed on sterile agar slants until the nymphs emerged. The aseptic nymphs were reared for as long as four months on sterile nutrient agar slants, with whole-wheat flour, yeast extract, and blood added. Attempts were made to cultivate the symbionts from these aseptic nymphs, but no bacterial growth appeared on any medium used in the 25 trials.

**DISCUSSION AND SPECIAL CONSIDERATIONS**

Several times during the course of this work, I felt certain that I had at last cultivated the roach bacteroid. First, there was an unidentified gram-negative, nonsporulating rod that occurred in nearly 50 per cent of the inoculated plates for several dissections, but after a change of cages and food supply for the animals, the prevalent bacteria suddenly became *Serratia marcescens*. After a considerable refinement of technique, the prevalent bacteria, occurring in nearly 10 per cent of inoculations, was a gram-positive, sporeforming rod that answered the description of *Bacillus cuenoti* as well, at least, as did the organism cultivated by Gropengiesser (1925). With greater precautions in sterilization and dissection of roaches and oothecae, however, the occurrence of this sporeforming rod was gradually reduced to very infrequent intervals, no matter what medium was used. Obviously, then, this sporeforming rod was not the bacteroid, or it would grow regardless of more careful manipulation.

As the technique was refined to eliminate the counterpart of *Bacillus cuenoti*, some very slow-growing diphtheroids occurred on subcultures from apparently sterile spots, usually on blood media. Usually they appeared about the same time on the plate from which the subculture was made. These diphtheroids fitted the description of Glaser’s *Corynebacterium periplaneta* very well: they were barred, gram-positive; sometimes pleomorphic; did not liquefy gelatine; utilized glucose, sucrose, and maltose without gas formation; grew slowly at first, doing well only on blood media, and gradually became adapted to routine culture media. These bacteria never occurred in more than 6 per cent of the subcultures from any set of inoculations, and sometimes 30 plates were inoculated and the regular 120 subcultures made from them with no organisms appearing except a stray yeast, a sarcina, or a slow-growing gram-negative bacillus. Strangely enough, the fewest bacterial growths appeared in the cultures from the series of dissections done under the most nearly optimum conditions.

The explanation for the diphtheroid cultures came accidentally one day when an air-control plate of blood medium was being examined under the microscope for the preliminary identification of colonies. On that plate were found two very tiny, nearly transparent droplets, only about 0.1 mm in diameter. They had the appearance of the diphtheroid colonies that had been studied and actually proved to be such. Similar diphtheroid colonies were found on nearly every air-control plate used after that. More numerous colonies and more kinds of diphtheroids were obtained by planting plates of Glaser’s blood media in se-
cluded spots in various offices in the Harvard Biological Laboratories. Other diphtheroids were isolated from the exterior and from the gut of the cockroach in cultures that were not too quickly covered by spreading colonies of more hardy forms. Numerous cultural tests failed to differentiate the diphtheroids of the experimental cultures from those of the air-control cultures, so the only conclusion that can be drawn is that the diphtheroids in the experimental cultures are contaminants that are peculiarly favored by the blood media and spotting technique used by Glaser. These diphtheroids, that seem to be everywhere, produce such tiny colonies in the original culture that it is almost impossible to distinguish them from fat droplets on the inoculated area, but when they are transferred to fresh media, they make enough growth in a few days to be readily visible. They probably enter the plates in the first place as air contaminants, because occasionally numbers of colonies of these diphtheroids were found on control plates left open under the supposedly sterile hood. The spotting technique, which necessitates opening the plate several times, is especially favorable to such airborne contaminants, and the procedure of subculturing apparently sterile spots makes visible these otherwise unnoticed colonies.

No attempt has been made to explain the presence, cultural behavior, or taxonomic position of these diphtheroids since it is quite evident that they are not the bacteroids of the cockroach. Some of them, however, were culturally indistinguishable from Corynebacterium periplaneta, Glaser.

In the results of these cultivation experiments, one fact is emphasized: that by any one special technique, one or a few kinds of bacteria are favored, resulting in partial or total elimination of other types of contaminants.

Glaser (1930) attempted to prove that the diphtheroids cultivated by him were identical with the "symbionts" by injecting suspensions of the diphtheroids into living roaches. Heavy suspensions of the diphtheroids did not kill them; therefore, he concluded, the roach must have a special natural immunity to this diphtheroid. During the course of this study I have determined by the inoculation of considerable numbers of roaches (partial results in table 3) that rapidly growing bacteria will kill the insects quickly even though the original injection is very small, although a thousand times as many organisms of a slow-growing strain, such as the diphtheroids or some yeasts, will not kill. This points only to the fact that these cockroaches are able to destroy relatively small numbers of not too virule bacteria and is proof neither for nor against the identity of the diphtheroids.

Mercier (1907), after preliminary examination, assumed that the "normal" cockroach oothecae are free of contaminating microorganisms. Gropengiesser (1925), Glaser (1930), and Bode (1936) accepted this assumption and used it as evidence that the bacteria they cultivated from the oothecae were the "symbionts." These workers overlooked the possibility of frequent inclusion between the oothecal lips of normal saprophytic organisms from the vagina or oviducts of the cockroach. Although this incidence of infection is not high (20 to 25 per cent in the animals used in these experiments), it is frequent enough that it cannot be overlooked. Conceivably in some stocks many more females could carry
such infection of a nature harmless to the roach but very disconcerting to med-"dlesome, bacteriologists. The high correlation in frequency and kind of organisms cultivated from entire oothecal contents and from the lips of the ootheca indicate that most of these infections came from the vagina, were at first limited to the region beyond the tips of the eggs (figure 3), and occasionally spread into the space around the eggs. Less than half as many contaminations were encountered following the described technique when fresh oothecae were used as the source of inoculum than when embryos of over ten days’ development were used. There was, however, no appreciable difference in frequency of positive growths between new and old ootheca when the entire capsule was macerated. Techniques in which the oothecae are macerated or are opened by separation of the lips get the advantage of all possible oothecal infections. This probably accounts for most of the positive cultures of roach “symbionts” from oothecae.

As for the other reported positive cultures of roach symbionts, little need be said. Mercier (1907) and Gropengiesser (1925) did not perfect their technique. Bode (1936) showed quite clearly that Bacillus cuenoti is not the roach symbiont by failing to cultivate anything from aseptically raised roaches, by failing with hanging drops, and by getting B. cuenoti only in liquid media with large amounts of inoculation material, in which case he greatly increased the chances of contamination and lost all chance of control or even of seeing what was happening. Yet, for some reason, not made clear in his report, Bode maintains, doubtfully, that Bacillus cuenoti is the roach “symbiont.”

TABLE 3
Injection of roaches with bacteria

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NUMBER OF ORGANISMS INJECTED</th>
<th>EXPERIMENTAL ANIMALS</th>
<th>ANIMALS SURVIVING 3 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serratia marcescens</td>
<td>100,000</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>400,000</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Tetragenous sp.</td>
<td>1,500,000</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Sarcina sp.</td>
<td>3,000,000</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diphtheroid I</td>
<td>2,000,000</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Diphtheroid II</td>
<td>5,000,000</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>15,000,000</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

Various bacteria isolated from the roaches themselves, grown on agar slants 24 to 48 hours, and suspended in Belar’s solution, were injected into last instar P. americana through the fovea of the femur in quantities of 0.05 to 0.15 ml. Numbers of bacteria in the suspension were determined by counting in a hemacytometer. Death from injected bacteria usually occurred within 20 to 60 hours, if at all, depending on virility and dose.
Mercier, Gropengiesser, and Bode did the thing that has been done too often in the cultivation of intracellular "symbionts": i.e., they depended on the accuracy of their technique and on the improbability of a constant contaminant as proof that the organism cultivated was the true "symbiont" (for other such examples see Schwartz, 1935) in spite of the fact that this organism (Bacillus cuenoti) is motile, sporeforming, stains solidly, and is strongly gram-positive, all of which characteristics contrast violently with the bacteroids in the cockroaches. Certain bacteria are known to change some of their characteristics under changed conditions, but all such drastic changes as this should be seriously questioned before being accepted. Glaser (1930) relied not only on technique but on striking similarities (general form, barring, nonmotility, and nonsporulating properties) between the cultivated organism and the roach symbionts, and in addition attempted serological comparisons. Hoover (1945), possibly, has hit upon one of the factors that has been greatly responsible for repeated failures in cultivation; i.e., time. Whether or not she has actually cultivated the symbiont of Cryptocercus is not at all certain from her report. From the results of the series of experiments described above, it is probable that all of these cultures are contaminants, and not the bacteroids.

Failure to cultivate the bacteroids of the roach may be due to any one of the following factors or a combination of these, and should in no way be interpreted to mean that these bacteroids are not living units:

1. These bacteroids may be so highly specialized for intracellular existence that they will not grow in any other medium. Many parasites, even of higher types, have not yet been grown outside their chosen habitats. These bacteroids are possibly more like rickettsiae than like bacteria, or, as Wallin (1925) suggested, perhaps the "symbiont" has become part of the host. Certainly they are adapted to a very specific set of conditions, and they cannot reasonably be expected to grow under conditions that do not closely approximate their normal habitat in most fundamentals.

2. The proper medium may not yet have been developed. We know very little definitely of the conditions in which these bacteria live such as osmotic pressure, specific salt, protein, and fat concentration; hydrogen ion concentration; and other conditions existing within the cell. There are many other bacteria and rickettsiae known, both saprophytic and pathogenic, that require special media. Some common pathogens have only recently been cultured by using new developments in media and by utilizing the living medium of chick embryos. It is yet possible that the proper medium may be developed either by trial and error or by careful analysis and duplication of the normal bacteroid habitat.

3. These bacteroids may have a normal reproductive cycle that is too slow for our culture methods. Other work now in preparation for publication shows that during the stage of most rapid increase of bacteroid numbers (late embryo) it takes 10 days to double the number. Unless this reproductive rate can be drastically increased, cultivation of the bacteroids by ordinary bacteriological methods is unlikely. Schwartz (1924) and Glaser (1930) hypothesized a chemical control of the host over its "symbiont" numbers, and they think that
this controlling element must be diluted before the “symbionts” can be cultivated. Contrary to their belief, I have found the “symbiont” increase to follow quite closely the weight increase of the host, and no factor controlling the bacteroid numbers in the cockroaches other than the regular factors controlling cell division and growth in the host could be detected. Theoretically, it is possible to adapt the “symbionts” to artificial media that would be so much more favorable for their development that they would make a really appreciable growth in a month or two.

There is no evidence to support the idea advanced by Mercier (1907) and reiterated by Gropengiesser (1925) that a yeast may at times displace the bacteroids. At times, however, the bacteroids in poorly fixed and insufficiently stained sections have somewhat the appearance of yeast because of swelling and vacuolization (figure 4). As Buchner (1930), Fraenkel (1921), and Bode (1936) have failed to find a yeast in the roach tissues, it is possible Mercier and Gropengiesser misinterpreted poor material. Sarcinae occurred frequently in the cultures in this series, as in those of Mercier (1907) and Gropengiesser (1925). These, as well as the yeasts, are probably saprophytes or temporary parasites that at times live in the vagina or oviduct and from there may be enclosed within the ootheca. There is, at present, no basis for hypothesizing that these organisms play a role as secondary “symbionts.”

Neither has anyone produced any evidence to support the contention (Buchner, 1930) that the bacteroids of cockroaches may be different organisms in different localities or under different conditions. Extensive study of many species of cockroaches and of the same species from widely separated localities (Kansas, Indiana, Florida, and Massachusetts) forces the conclusion that the intracellular symbionts of the cockroaches are as specific and constant as are the roaches themselves. In this series of studies, there is no indication other than that each species of cockroach is the specific host for one organism, and that one type only may live within any one roach bacteriocye. Roaches of all ages of several species (Periplaneta americana, P. australasiae, Blatta orientalis, Blatella germanica, Paroblatta pennsylvanica, P. uheriana, P. virginiana, P. lattra, Nyctobora noctivaga, Buryctos floridana, and Cryptocercus punctulatus) were carefully studied for occurrence, behavior, morphology, and staining reactions of symbionts. Most of these species were used both fresh from the field and after long maintenance under laboratory conditions. Many individuals were subjected to drastic experimental conditions, such as partial and complete starvation; unbalanced diet; extreme temperature variations; injections of yeasts, bacteria, and chemicals into the hemocoel; X-ray and ultraviolet treatment; and deliberate neglect resulting in overcrowding and accumulation of their own wastes. In all cases, the morphological and staining characteristics of the symbionts remained remarkably constant, and indicate that the symbionts are at least all within the same genus, the differences being merely slight average variations in size and in arrangement of bars. Only extreme variations in the symbiont of any roach species can be distinguished from any other. Paroblatta symbionts are a little thinner (0.8 μ as against 1 to 1.1 in other genera); Blatta symbionts
have a greater tendency to form chains of three or four rods, and are more noticeably curved; *Blatella* symbionts are more uniform in length; and *Cryptocercus* symbionts have broader dark bands and fewer light-staining areas than are usual in the others. I have not found the extremely slender symbionts described by Hoover (1945). These variations are so slight that I cannot definitely identify any host by study of a film preparation of symbionts.

As to the taxonomic position of the cockroach symbionts, I have only to offer that they are generically all the same. Their morphology and staining reactions do not definitely place them in any established genus of bacteria. They are gram-positive but not strongly so. They are uniform rods with rounded ends, straight to half-moon curve, that vary in length from 1.5 to 6.5 μ in the same host. They divide by fission, typically into two unequal rods that tend to remain united until, or past, the next division time. They are not acid-fast, and form no spores even after the death of the host. They show a barred pattern with some stains, especially with the Giemsa stain and haematoxylin, and in some cases with carbolfuchsin and with Gram's stain, and to a much less extent with Alberts, Neisser's, and Loeffler's stains. No cilia have been demonstrated and no movement of the symbionts has been observed by me or recorded by others. These symbionts, then, have some of the characteristics of *Corynebacterium*, of *Spirillum*, and of *Bacterium*.

The barred appearance of intracellular "symbionts" following Giemsa stain or haematoxylin seems to be rather common as it has been specifically mentioned in the gut "symbiont" of *Rhodnius* (Wigglesworth; 1936); in the "symbiont" of the bedbug mycetocyte (Buchner, 1930); in the ant "symbiont" (Lilienstern, 1932); in the root nodule "symbionts" of legumes; and possibly in others that have escaped my attention. Of these, only the "symbiont" of *Rhodnius* has been designated by the investigator as a diphtheroid. Possibly this disposition of materials within the "symbiont" body is a characteristic derived from the intracellular existence rather than an indication of relationship to the *Corynebacterium*. It would probably be desirable for taxonomists so to define the *Rickettsiaceae* (as proposed by Steinhaus, 1940) as to include most of the bacteria-like intracellular symbionts, at least until they can be demonstrated to belong to the *Bacteriaceae*.

Glaser's species of diphtheroids (*Corynebacterium periplaneta*) is probably a valid species, if we understand that it has not been demonstrated to be the intracellular symbiont of *Periplaneta*.

**SUMMARY AND CONCLUSIONS**

Various techniques for manipulations of intracellular bacteroids, and all kinds of media on which growth of the intracellular bacteroids of cockroaches have been reported, were tried.

Numerous attempts to cultivate the intracellular bacteroids of *Periplaneta americana*, *Blatta orientalis*, *Paroblatta pennsylvanica*, and *Cryptocercus punctulatus* failed.

All bacteria suspected of being the bacteroids were definitely eliminated by
reducing their frequency of occurrence with each refinement in technique, and by cultivating their counterparts from other sources.

Hanging drop cultures, tissue cultures, implants on chick chorioallantois, and injections of the bacteroids into chick amniotic cavities—all failed to produce any perceptible increase in bacteroid numbers.

It is improbable that yeasts or cocci ever displace the normal bacteroids of the cockroach.

It is very doubtful that anyone has yet cultivated the intracellular bacteroids of any cockroaches.

The intracellular bacteroids of the blattids studied are generically the same and should probably be included in the Rickettsiaceae.

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