Preliminary Studies on the Bacterial Cell-mass (Accessory Cell-mass) of Calandra oryzae (Linn.): The Rice Weevil.

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With Plates 22, 23, and 4 Text-figures.

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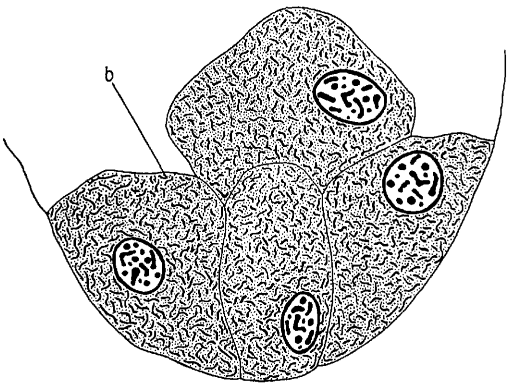
1. Introduction.

In my previous work on Calandra (1927) reference was made to the presence in the larval stage of a mass of cells between the nervous system and the alimentary canal in the region where the fore-gut passes into the mid-gut. On account of its

Text-fig. 1.



Portion of a section through a mesenteric caecum showing bacteria, *b*, within the ' bacterial cells '. × 930.

future anatomical relation to the mid-gut of the adult, this mass was referred to as the ' accessory cell-mass '.

More work on the subject revealed the fact that the cells of this mass contain in their cytoplasm numerous micro-organisms which proved to be bacteria (*b*, Text-fig. 1).

This mass of cells will therefore be referred to as the ' alimentary bacterial cell-mass ', and its cells as the ' alimentary bacterial cells ', in order to distinguish them from similar cells at the tips of the ovarioles to be described in the course of this paper and to be referred to as the ' ovarian bacterial cells '.

Pierantoni (1927) merely refers to the ' accessory cell-mass ' of the present author as an ' organo symbiotico ' with no evidence concerning the exact relation between the micro-organisms contained within the cells of this organ and the weevil in question. His account seems to be based mainly on my previous work. He only describes the mass under consideration as being paired in the larva. For the mere fact that this mass is saddle-shaped in some sections certainly suggests a paired nature; but, when a whole series of sections is examined, there is not the slightest doubt that the cells in question are in one mass.

In this paper it is only proposed to give a preliminary account of the structure of these intracellular bacteria, their mode of transmission from one generation to the next, and their activity during the life of their host.

II. Technique.

The developmental history of the bacterial cells was best studied in material fixed in Carl's fluid and stained in Delafield's haematoxylin (Mansour, 1927).

For the study of the bacteria in the cells and tissues of their host, Schaudinn's fluid proved the most suitable fixative. The organs to be studied were dissected out by means of very fine needles and transferred to the fixative for 3–5 minutes. They were then washed thoroughly in 50 and 70 per cent. alcohol, dehydrated, embedded, and sectioned in the usual way.

Smearing before fixation was also found useful for the study of the general structure of the bacteria. After drying up, the fixative was applied for about 5 minutes, then the smear was washed under the tap, dried, and stained.

Giemsa stain as used by Minchin[1] and Gram's as recommended by Eyre[2] gave very good results. Alkaline methylene blue was also found suitable for smear preparations.

[1] ' The Microtomists' Vade-Mecum ', London, 1928.

[2] J. W. H. Eyre, ' Bacteriological Technique ', Philadelphia and London, 1916.

III. The Intracellular Bacteria.

The intracellular bacteria are in the form of bacilli rounded at both ends and frequently joined together in strings, some being as much as 50μ long (Text-fig. 4). The separate bacilli are motile and vary from 3μ to 5μ in length and are about $0{\cdot}6\mu$ wide. They stain well with Giemsa, both in

Text-figs. 2–4.

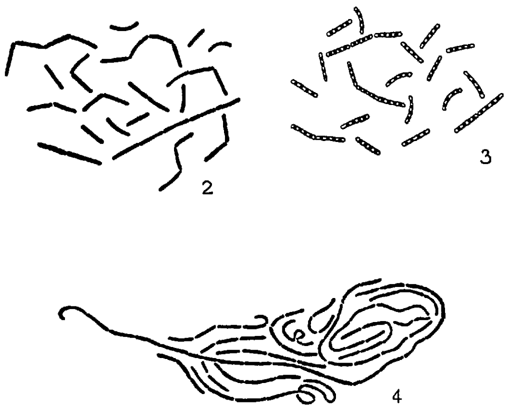


Fig. 2.—From a smear preparation of the mid-gut of an adult. Giemsa.

Fig. 3.—From a smear preparation of the mid-gut of an adult. Gram.

Fig. 4.—From a smear preparation of the ‘alimentary bacterial cell-mass’ of the larva. All × 2660.

sections and smears, showing a uniform rod-like structure (Text-fig. 2). With Gram's, to which they are positive, they exhibit a distinct beaded structure (Text-fig. 3). Smears from the various stages of the host show that the strings are longer and more numerous in the egg, larva, and pupa : on the other hand, they are shorter and the presence of separate bacilli is more frequent in the prepupal and adult stages. This is probably due to the fact that in the egg, larva, and pupa, the bacteria being comparatively inactive do not separate from their chains. In the prepupa, the breaking up of the chains into bacilli may be connected with the migration of the ' bacterial cells ' to their final position around the developing mid-gut. Finally, in the adult, the bacteria themselves show great activity, and pass from their host-cells in the form of bacilli into the lumen of the alimentary tract (see p. 426).

In the larval stage the arrangement of the bacteria within the cells is very characteristic. Examination of smears shows that the long strings are coiled round one another in a peculiar fashion (Text-fig. 4). This appears to be due to the crowding of the strings within a limited space.

Artificial cultivation has not so far been attempted.

IV. The ' Alimentary Bacterial Cell-mass ' in the Larva and during Metamorphosis.

The first conspicuous appearance of this mass, the mode in which it takes its place during the larval stage, its fate during metamorphosis, and the position of its cells in the adult, have been all described in the course of my previous paper.

Throughout the larval stage the bacteria within the cells are comparatively inactive and the ' mass ' is to be found ventral to the gut and separated from the digestive epithelium by the muscular wall of the latter. Examination of the larva shows that the digestive epithelium and the food material inside the alimentary canal are free from the characteristic bacteria contained within the cells of the ' bacterial cell-mass '.

During metamorphosis the ' bacterial cells ' are arranged round the developing mid-gut epithelium and ultimately form the outer walls of the anterior mesenteric caeca.

V. The Intracellular Bacteria during the Adult Stage.

Contrary to their behaviour during the larval stage, the intracellular bacteria are very active during the imaginal life.

They pass in large compact ball-like masses into the lumena of the respective mesenteric caeca, and thence to the cavity of the mid-gut, where they infect some of the epithelial cells (fig. 1, Pl. 22). The bacteria in a newly infected cell grow actively and form a spherical mass (*m b*, fig. 2, Pl. 22) within the cytoplasm, which in this region stains more lightly than the rest of the cell. Eventually these masses pass into the lumen of the mid-gut (fig. 3, Pl. 22) in a similar fashion to that described for the original ' bacterial cells '.

VI. Distribution of the Free Bacteria in the Alimentary Tract of the Adult.

From the anterior portion of the lumen of the mid-gut the liberated bacteria which are in the form of bacilli spread anteriorly and posteriorly into the fore and hind guts respectively. In the gizzard they mix with the food of their host and apparently grow on it. Their number decreases gradually forwards. In the hind-gut they are present in large quantities mixed with the indigested food. In the anterior portion of this region of the gut they are very numerous indeed, but they become scarce posteriorly. The decrease in number of these bacilli seems to be correlated with the appearance of a coccus form ; and in the crop and the hind portion of the proctodaeum where the bacillus form is very scarce, the coccus form is found in large quantities.

It is known that a bacterium may vary greatly under different conditions (Mercier (1907) and Eyre [1]), and as the conditions within the cells are totally different from those in the lumen of the gut, it seems not unreasonable to suggest that the bacterium under consideration, when intracellular, is always rod-shaped, but when growing on the food of its host assumes after a short time a more or less rounded form.

Examination of the faeces of the adult supports this conclusion. It has been mentioned above that the bacillus form is

[1] Footnote, p. 423.

found in large quantities in the anterior half of the proctodaeum. In the faeces this form is practically absent, while the coccus form is very prevalent and forms a considerable portion of the faecal matter. This shows probably that by the time the indigested food is ready•to be passed out, most of the bacilli have assumed the coccus form.

In the preparations examined no sign of disintegration or digestion of these bacteria has been observed. It is probable, therefore, that after being liberated from the cells, the bacteria live for a short time on the food taken in by their host and pass to the exterior mostly in the form of cocci.

VII. The Relation between Calandra and its Intracellular Bacteria

Great importance has been attached recently to the presence of intracellular organisms in the alimentary tract of certain insects, especially in those with cellulose feeding habits. Buchner (1928 a), Pierantoni (1927), and others assume that such micro-organisms render valuable nutritive services to their hosts and describe the relation as being symbiotic. In the light of the present work there seems to be a doubt as to the accuracy of this assumed function of the micro-organisms.

The larva of Calandra, being inside the grain throughout its life, feeds entirely on the internal contents, i.e. starch and proteid-grains. It has been pointed out (p. 425) that the intracellular bacteria here are not found within the alimentary tract, and there seems to be no doubt whatsoever that the nutritive material the larva needs is digested with the aid of enzymes secreted by the digestive epithelial cells.

The adult, on the other hand, feeds inside the grain only for a very short period after its eclosion. It then bores its way out through the pericarp and the testa and lives the rest of its life outside the grain. Its food is similar to that taken in by the larva. It differs in the slight amount of cellulose eaten up during the emergence from the grain and again during the attack of fresh material. This amount of ingested cellulose taken in is quite small and a very similar quantity has been observed in the faeces ; so that the question of cellulose digestion here is of minor importance. Thus the food of the adult is practically similar to that of the larva and consists mainly of starch and proteid-grains.

Uvarov (1929), in his masterly summary of the literature on ' Insect Nutrition and Metabolism ', has pointed out that proteases and diastases occur in the digestive fluids of all insects studied. It is quite probable that the larva of Calandra, too, possesses similar enzymes for digestion. As the mid-gut of the adult Calandra is ectodermal in origin as is that of the larva, it is quite conceivable that the digestion in the adult is similar to that in the larva without the help of the intracellular bacteria it harbours. This conclusion holds good unless it is to be assumed that the digestive epithelium of the adult lacks certain enzymes, or is supplemented by certain enzymes due to the activities of the bacteria. At present there is no evidence at all to support such an hypothesis.

Hylobius abietis, which contains similar ' bacterial cells ' (Mansour (1927) and Buchner (1928 *a*)), illustrates more clearly the doubt which the present author entertains concerning the supposed role of such intracellular bacteria. As in Calandra, the intracellular bacteria are comparatively inactive in the larval stage. During metamorphosis the ' bacterial cells ' arrange themselves round the developing digestive epithelium ; probably in the adult Hylobius they behave in a similar manner to that described for Calandra. The larva and adult feed on different food materials. The larva feeds inside the old pine stumps and takes in large quantities of wood. The adult, on the other hand, feeds on young pine shoots and needles and probably does not touch hard wood at all. If the intracellular bacteria in Hylobius help in the digestion of wood, as has been assumed by many authors, one would expect them to be active in the larval stage as well.

In conclusion, however, it must be admitted that the role of the bacteria in the gut of Calandra is obscure. What the relation between the two organisms—bacteria and Calandra—may be, no one really knows. Under these circumstances, to describe the behaviour of the bacteria and Calandra as symbiotic is premature, misleading, and unjustifiable.

VIII. Transmission of the Intracellular Bacteria from one Generation to the Next.

(*a*) The Female Genital Organs and the 'Ovarian Bacterial Cells'.

The infection of a new generation takes place in the ovarioles. There are two ovaries, each consisting of two acrotrophic ovarioles which open into a common oviduct. The two oviducts, one from either side, open into the uterus, which leads into the vagina. The anterior extremity of the latter is expanded to form the bursa copulatrix. Into the dorsal surface of the vagina, near its posterior end, opens a very narrow tube which leads into the horseshoe-shaped thickly chitinized receptaculum seminis.

Examination of an ovariole discloses the presence at its tip of a group of ' bacterial cells ' similar to those surrounding the mesenteric caeca of the adult. These ' bacterial cells ' are closely associated with the germarium and are enclosed within the delicate membrane investing the whole ovariole. In male gonads such ' bacterial cells ' are absent.

(*b*) Infection of the Egg.

During oogenesis the bacteria leave their host-cells (fig. 4, Pl. 22) and pass backwards into the germarium, where they are to be found in the nutritive fluid scattered in between the developing oocytes and the nutritive cells. Growing eggs in the germarium are found to be infected (fig. 5, Pl. 22). In all the subsequent stages of growth of the ovarian egg similar infection has been observed in the cytoplasm (fig. 6, Pl. 22). Within the cytoplasm this infection remains and when the yolk is deposited the bacteria are to be found scattered in between the globules (fig. 7, Pl. 23). The bacteria remain throughout the early stages of embryonic development in a similar position.

In some females the genital ducts were found to contain practically pure cultures of similar bacteria; in all the males examined no bacteria were seen in the corresponding organs. Further, it has been observed that masses of bacteria pass down among the growing oocytes into the uterus. So the balance of evidence seems to point to the fact that the bacteria present in some female ducts come down from the tips of the ovarioles. It seems that owing to the large number of bacteria present in the region of growing eggs, the infection of the uterus is direct and not through the exterior genital aperture from the proctodaeum as Buchner (1928*b*) assumes for Hylobius, Otiorrhynchus, and the great majority of infected Curculionids.

The bacteria in the genital ducts of the female of Calandra take no part in the infection of the eggs.

IX. The Bacteria during Embryonic Life.

(*a*) Formation of the 'Alimentary Bacterial Cell-mass'.

The formation of the 'alimentary bacterial cell-mass' ('accessory cell-mass') has been dealt with previously (Mansour, 1927). It need only be mentioned here that the change in the cytoplasm of the cells forming this mass is due to the invasion of bacteria from the surrounding yolk-mass.

The 'bacterial mass' in question appears in all developing eggs and is present in all larvae. It is destined to infect the alimentary canal of the adult.

(*b*) Formation of the Genital Rudiments and the Appearance of the 'Ovarian Bacterial Cells'.

The genital rudiments are paired. They are differentiated at a very early stage in the embryonic development. They first appear as a mass of cells situated at the posterior end of the egg in between the yolk-mass and the blastoderm. Towards the end of the embryonic life these rudiments are situated in the dorsal region of the posterior half of the embryo. They retain this relative position throughout the latter part of the embryonic period and the whole of the larval stage.

Examination of a number of genital rudiments in late embryos and larvae shows that two types can be distinguished, one associated with few bacterial cells (*ov b c*, fig. 9, Pl. 23), and from its developmental history giving rise to ovarioles, the other free from such cells and giving rise to testes (fig. 8, Pl. 23).

The rudiments destined to give rise to ovarioles (fig. 9, Pl. 23) show bilateral symmetry and each half consists of a small mass of ' bacterial cells ' (*ov b c*), a mass of germ-cells (*fe g c*), and a few basal cells with deeply staining nuclei (*bs c*).

The mode of infection of the ovarian rudiments has not so far been followed in detail. Apparently some of the cells associated with these rudiments, while still close to the yolk-mass, are invaded by bacteria coming from the inner cytoplasm of the egg.

X. Formation of the Ovarioles during Metamorphosis.

Every ovarian rudiment gives rise to two ovarioles and an oviduct. The basal cells grow backwards in the form of a V-shaped tube, while the other constituents of the rudiment are separated into two equivalent sets, each set consisting of a mass of germ-cells and a group of ' bacterial cells '. At this stage the developing ovariole is club-shaped (fig. 10, Pl. 23). The germ-cells divide actively and block the cavity of the tube, whose walls become very thin and form the inner coat of the ovariole (*i c d o*, fig. 11, Pl. 23). The bacterial mass increases slightly in size and remains all the time at the anterior extremity. From the point of junction of the developing ovarioles, the oviduct grows backwards to meet the other genital ducts, which in the meantime have been developing inwards from the hypodermis, and by the time metamorphosis is completed, the oviducts are found continuous with the uterus.

XI. Mode of Transmission of the Intracellular Bacteria in other Infected Curculionids.

Masses similar in appearance and in behaviour during metamorphosis to the bacterial mass of C. oryzae have been described by the present author (1927) in C. granaria and Hylobius abietis. In the case of C. granaria it has been ascertained that the mode of infection of the egg is similar in all respects to that described for C. oryzae.

Similar ' bacterial masses ', probably behaving in a similar fashion, were also described by me in Odioporus glabricollis and Rhyncolus lignarius.

Examination of the larvae of Rhyncolus lignarius discloses the fact that the genital rudiments are of two kinds, one with cells similar to those of the infected mass, and the other free from such cells. Probably further work would prove that the rudiments with infected cells give rise to ovarioles, and that the mode of infection is similar to that which has been described for Calandra.

Buchner (1928 *a*) independently confirms the present author's observations on Hylobius, and describes similar masses in Otiorrhynchus inflatus, Pissodes notatus, Cryptorrhynchus lapathi, Cionus sp., Sibina pellucens, Protapion aeneum, and Cleonis sp.

With regard to the mode of infection of the eggs of these Curculionids, Buchner describes two methods: one for the family Cleonidae, and the other for the rest of the infected Curculionids, with especial reference to Hylobius abietis and Otiorrhynchus inflatus.

With regard to the mode of infection in the Cleonidae, Buchner (1928 *a*, pp. 41–3) writes : ' So far I have found the counterpart of the fungus syringes in Siricidae, Cerambycidae, and Anobiidae only in the Cleonidae—it takes the form of bacterial syringes. At the place where the last segment joins the penultimate and which in the position of rest is withdrawn into the preceding segment, the narrow orifices of a long club-shaped organ lie on both sides. On crushing the syringe endless masses of slender symbiotic bacteria are squeezed out. We have not yet had the opportunity of observing the function of these syringes during oviposition, so that we cannot decide whether clumps of bacteria issuing from the orifices and sticking to the egg surface are eaten by the young larva, or whether the bacteria make their way in through the micropyle, and are thus introduced into the next generation much earlier.'

He describes and figures four large 'Bakterienorgane', apposed to the wall of the anterior portion of the mid-gut of Cleonus, but admits that in the adult he has not been able to demonstrate the presence of 'bacterial cells' along the mesenteron. Evidently these masses are not to be compared with those of Calandra, Hylobius, &c., and need not concern us here.

The case of Hylobius and Otiorrhynchus, on the other hand, raises important points.

With reference to the mode of transmission in these two insects, Buchner (in the same work, p. 44) mentions: 'So far, it is only in Hylobius and Otiorrhynchus that I can say that transference takes place in such a simple and ingenious way. Here I find, in the large bursa copulatrix, considerable quantities of bacteria near and between the sperm masses, and I assume that they enter the micropyle of the weevil's egg with the spermatozoa. Evidence for this must be sought by further difficult research.'

The same author (1928 *b*, p. 32) adds that in the great majority of weevils this is evidently the original mode of infection of the egg, and assumes that the bacteria in the genital ducts of the female come from the proctodaeum through the anus and the external genital aperture. He also mentions, without giving evidence for such a statement, that the ovarian eggs are not infected, and that the fate of these bacteria during embryonic development has not yet been determined.

It has been pointed out (p. 430) that similar bacteria are found free in the female genital ducts in the case of Calandra and that these organisms pass downwards from the tips of the ovarioles. It may be that the bacteria in the female genital ducts of Hylobius and Otiorrhynchus have a similar origin. As there is no evidence at all for Buchner's assumptions that the bacteria enter the egg with the sperms, it is probable that further work on Hylobius and Otiorrhynchus will prove that the mode of infection is similar to that which has been described for Calandra.

XII. Summary.

1. The bacteria within the 'bacterial cells' of Calandra are in the form of bacilli.

2. The bacilli do not pass into the alimentary tract of the larva.

3. In the adult the bacilli pass from their host-cells into the lumen of the gut, mix with the food there, and pass out with the faeces mostly in the form of cocci.

4. The relation between Calandra and the intracellular bacteria is obscure and so far cannot be described as symbiotic.

5. 'Bacterial cells' have been found at the anterior tips of the ovarioles.

6. The ovarian eggs are invaded at a very early stage during their growth by bacteria coming from the 'ovarian bacterial cells'.

7. The bacteria remain in the cytoplasm of the egg scattered in between the yolk globules throughout the early embryonic life.

8. In all developing eggs the 'alimentary bacterial cell-mass' appears during the latter part of embryonic life.

9. In the eggs destined to give rise to females the genital rudiments are associated with 'bacterial cells'.

10. The developmental history of the 'ovarian bacterial cells' has been followed out.

XIII. Acknowledgements.

I desire to express my best thanks to Professor Victor Jollos (formerly of the Egyptian University) for his advice concerning technique.

I am further greatly indebted to Dr. E. J. Allen for kindly allowing me to finish this work at the Laboratories of the Plymouth Marine Biological Station.

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EXPLANATION OF PLATES 22 AND 23.

All figures are from camera lucida drawings.

List of Reference Letters.

b, bacteria or bacterium ; *bs c*, basal cells ; *cy*, cytoplasm ; *f c*, follicular cells ; *fe g c*, female germ-cells ; *ft c*, fat cells ; *i c d o*, inner coat of the wall of developing ovariole ; *ma g c*, male germ-cells ; *m b*, mass of bacteria; *mg ep*, mid-gut epithelium ; *mus w*, muscular wall ; *n*, nucleus ; *nl*, nucleolus ; *o c d o*, outer coat of the wall of developing ovariole ; *ov b c*, ' ovarian bacterial cells ' ; *y g*, yolk-globule.

Plate 22.

Fig. 1.—Portion of a transverse section through the anterior region of the mid-gut of an adult showing infected epithelial cells. Schaudinn and Giemsa. × 600.

Fig. 2.—An epithelial cell with a mass of bacteria (*m b*) within the cytoplasm. Schaudinn and Giemsa. × 800.

Fig. 3.—Portion of a transverse section through the anterior region of the mid-gut of an adult showing the passage of a mass of bacteria into the lumen of the gut. Schaudinn and Giemsa. × 800.

Fig. 4.—Portion of a section through the anterior tip of an ovariole during oogenesis. Schaudinn and Giemsa. × 800.

Fig. 5.—A section through a very young oocyte while still in the germarium, showing bacteria (*b*) already in the cytoplasm. Schaudinn and Giemsa. × 900.

Fig. 6.—A section through an oocyte surrounded by follicular cells. The size at this stage is nearly half that of the fully grown egg. Schaudinn and Giemsa. × 480.