MYCETOMAL MICRO-ORGANISMS

ASSOCIATED WITH TWO WEEVILS

A STUDY OF THE ASSOCIATION BETWEEN TWO WEEVILS (*SITOPHILUS ORYZA* L., and *SITOPHILUS GRANARIUS* L.,) AND THE MICRO-ORGANISMS OF THEIR MYCETOMES

By

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SCOPE AND CONTENTS:

A biological investigation of the apparently intimate and mutually beneficial relationship existing between certain plant-like micro-organisms and certain insects.

The literature of the subject is briefly reviewed and discussed and a critical appraisal of previous work is made. A problem, the association of certain micro-organisms with two species of grain feeding weevils, is discussed in greater detail and a method of approach is proposed and analysed.

Studies of the micro-organisms in vivo and in vitro are described in conjunction with investigations, by experimental techniques, of the association of the micro-organisms and the weevils. Findings are discussed and conclusions are presented.

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CONTRIBUTIONS TO OUR KNOWLEDGE OF INSECT MICROBIOLOGY MADE IN THE PRESENT WORK

1. Considerable clarification of the whole complex relationship between Sitophilus oryza and Sitophilus granarius and their associated mycetomal micro-organisms has been achieved.
2. The misleading statement that Sitophilus granarius is free of mycetomal micro-organisms which has been made in several authoritative works (Jeannell, 1949; Roeder, 1953; Steinhaus, 1946; and Wigglesworth, 1950) has been corrected and it has been shown how this error was made.
3. The question of the micro-organisms being only cell particles has been considered very carefully and it has been contended that, in the present state of our knowledge, the evidence definitely supports previous authors who regarded the structures as micro-organisms.
4. For the first time micro-organisms associated in this way with insects have been studied by the recently developed technique of phase contrast microscopy, which permits the microscopical examination of living unstained material.
5. The behaviour and staining reactions of the micro-organisms have been described and the findings compared with the observations of others.
6. It has been found that, in the material available, it has been possible to distinguish the micro-organisms of one species of weevil from those of the other by a size difference.
7. It has been found that strains of S. granarius exist in Canada, most individuals of which are free or almost free of the standard mycetomal

micro-organisms. This finding has provided additional support for the contention of an earlier Egyptian worker who claimed that S. granarius existed in a micro-organism-free, Egyptian strain.

8. Evidence has been produced to show that the strains of weevils differing in their micro-organism population can be distinguished externally by certain general facies.

9. Experiments in which different strains have been crossed have produced evidence that the mycetomal micro-organisms are passed congenitally to the progeny through the female.

10. It has been possible to remove the micro-organisms from the insects to specially prepared slide cultures containing synthetic tissue culture media, where they remained apparently alive for many months.

11. Observations have strongly confirmed previous findings that the organisms produce spores by a process of proliferation.

12. Observations have suggested that a proportion of the spores germinate by more than one germ-tube. This and other characters have combined to suggest that the mycetomal micro-organisms in the insects studied are Actinomycetes.

13. It has been shown experimentally that the feeding of Terramycin-treated grain to the weevils affected S. oryza more adversely than S. granarius. Subsequently, it has been shown by microscopical examination that the Terramycin appeared to have an adverse effect on the micro-organisms.

14. It has been suggested that the evidence is in favour of believing S. oryza to be more dependent on its mycetomal micro-organisms than is S. granarius.

HYPOTHESES ADVANCED

1. It has been suggested that, while there is, at present no evidence of oral transfer of the mycetomal micro-organisms, the weevils originally became infected in that way.

2. The hypothesis has been presented that the mycetome is an endocrine gland which is subserving the additional function of harbouring micro-organisms during the larval life of the insect.

I. INTRODUCTION

(a) General and Historical Aspects

Insect microbiology is the study of the association of various micro-organisms with insects in various stages of their life histories. In recent years considerable attention has been paid to the study of insect microbiology. This is obvious from the works of Buchner (1953) and Caullery (1952) which contain extensive sections on insect microbiology, and those of Paillot (1933) and Steinhaus (1946) which are devoted entirely thereto. Insect microbiology moreover receives attention in many of the standard works in Entomology (e.g., Hagan, 1951; Roeder, 1953; and Wigglesworth, 1950) and microbiology (e.g., Bergey, 1948; Borden, 1950; and Waksman, 1950).

According to Steinhaus (1949), de Bary, in 1879, gave the name symbiosis to all associations between organisms including true parasitism. But since that time the word symbiosis seems to have come to mean to many biologists the association of two living organisms together for mutual benefit. Such close associations have long been a fascinating study to biologists and attempts have been made to classify the different kinds of association. (See, for example, Burkholder, 1952).

The American Association of Parasitologists has recently ruled that the word symbiosis should now be used as de Bary originally intended. The term mutualism should be used for those associations where there is mutual benefit. It seems doubtful if this has been accepted by

European workers in view of the present existence of the Paul Buchner Institut für experimentelle Symbioseforschung, at the University of Munich. It seems, indeed, that that degree of precision in the use of terms that is so desirable in any intellectual discussion is lacking. Thus it seems wiser to refer to the phenomena simply as associations. And herein that terminology will be adopted as far as possible. In much of the older work the terms symbionts or symbiotes have been used and these are often retained in the present account when referring to such work.

The associations between insects and micro-organisms seem to be of four main kinds. 1). The micro-organism may be harmful to the insect, as in many protozoal diseases (Musgrave and MacKinnon, 1938) or virus diseases (Steinhaus, 1949). 2). The micro-organism is not noticeably harmful to the insect nor apparently to any other organism (e.g. many of the gregarines found in insects. 3). The organism is not noticeably harmful to the insect but is harmful to some other secondary host, e.g. the malarial parasite in the mosquito. 4). The micro-organism is of benefit to the insect. It is with this fourth type of association that this thesis is particularly concerned.

There has been much interest during the past half century in the micro-organisms associated intimately with insects and thought to be of benefit to them. Such micro-organisms are, in some species of insects found in organs that seem to have been specially developed by the insect body to harbour them; in other species of insects they are found in some organ common to the whole class Insecta. Now, insects are well known for their diverse feeding habits and they are found in almost every ecological

niche of the terrestrial environment. Different species of insects can feed on such diverse substrates as dry flour, vertebrate blood and plant sap. Yet they seem to have many of the dietary requirements of other metazoa, including a need for some of the vitamins. It is thus not surprising that the opinion that insect nutrition is sometimes aided by micro-organisms has gained, in the course of years, no small degree of acceptance. Only comparatively recently, however, has there been any very clear evidence for this. (See Brecher and Wigglesworth, 1944: and Blewett and Fraenkel, 1944).

The micro-organisms that have been suspected of being of benefit to the insect host have been reported chiefly from the gut, the gonads and the fat-body, depending on the species of insect concerned. The organisms have been described as bacteria, yeasts, fungi, rickettsias and actinomycetes.

According to Caullery, Paillot and others, it was in 1850 that the interest in possibly beneficial micro-organisms can be said to have begun. In that year Leydig described a strange green body in aphids. In 1877 Putnam recognised that a similar organ in Coccids (Scale insects) contained micro-organisms. During the years various workers directed their attention to the phenomenon and some review will be found in the texts of Paillot and Buchner. But it was not until 1910 that any real advance in our knowledge seems to have been made. In that year the investigations of Pierantoni in Italy and Sulc in Bohemia were published. Sulc apparently invented the terms mycetome and mycetocyte. The mycetome was the organ in the insect that harboured the micro-organisms; it was built up of many separate cells called mycetocytes. Sulc, moreover,

referred to the organisms as symbiotes and believed them to be yeasts. He supposed that all yeasts from aphids belonged to only one species, Saccharomyces aphidis. Pierantoni believed that the rounded cellular inclusions of Homoptera were fungal elements and that they were hereditarily transmitted. It seems, too, that Pierantoni considered that the symbiotes helped the insects in the digestion of starches and sugars. The German worker, Buchner, who later became an acknowledged expert in this field of study, at first accepted the fungus or yeast-like nature of the micro-organisms, but later he described them as derived from bacteria. Paillot quotes Pfeiffer as finding that the symbiotes of the bedbug were of the most diverse forms, from those typically bacterial to those discoidal in shape. Later workers mentioned the occurrence of two symbiotes and of symbiotes and bacteria and of 'forms of passage'. Caullery (1952) in his book states that the symbiotes of each species are clearly defined. Even the number of mycetocytes is fixed. "There are some insects, as Buchner recognised, which possess two or several species of symbiotes". The host, writes Caullery, has control over the number of organisms which never get out of control. Mahdihassan (1951) has claimed that a close relationship exists between species of host and species of symbiote: Coccids producing much wax harbour yeasts, those producing little wax, bacteria. Scheinert (1933), Mansour (1927) and Tarsia-in-Curia (1933) have all claimed to have found evidence of the hereditary transmission of micro-organisms in weevils. Riess (1930) and Aschner and Riess (1932) have also provided evidence of hereditary transmission of micro-organisms in the body louse. They have, moreover, made significant contributions to our knowledge of the whole subject in

their work. The micro-organisms in the adult louse are held in a mycetome near the gut. Hereditary transmission takes place from the gut to the gonads and thence to succeeding generations (see Fig. 1 which is from Reiss). These investigators also attempted to determine what part, if any, these organisms played in the insects economy. The results of their experiments, involving extirpation of mycetomes and their displacement by centrifugalisation, are attractive. The implications were that extirpation of the mycetomes when full of symbiotes caused marked deficiency symptoms in the louse. "If, however, extirpation took place after the micro-organisms had migrated to the oviduct, it had no influence on the well-being of the insect." These investigators were, however, unable to provide evidence as to the exact function of the micro-organisms.

Glaser (1946) showed that the diphtheroid organism found in males and females of Periplaneta americana could be adversely affected or destroyed by either sulphathiazole, sodium or calcium penicillin or prolonged subjection to 39°C. The impression gained from the results was that the treatments prolonged the juvenile period of the roaches. It seemed that in the absence of the micro-organisms development of the female gonads was retarded or prevented but the male gonads were not affected.

Many attempts have been made to isolate beneficial micro-organisms from the insect hosts. Pierantoni and Peklo are both credited with isolating organisms (see Paillot, 1933). Brues and Glaser (1921) claimed to have isolated the beneficial micro-organisms from the scale insect, Pulvinaria innumerabilis. From their rather fragmentary observations it seemed that oval symbiotes multiplied and changed morphologically in

April and May. They could be seen in groups forming strings similar to mycelium. In cultures, the organisms stayed yeast-like for a day or two; after prolonged incubation on solid media the formation of a distinct mycelium always occurred. The mycelium was branched and of quite irregular form. The organism produced a proteolytic enzyme and a lipase. Later, in 1930, Glaser claimed to have isolated diphtheroids from the cockroach, Blatella germanica; and in the same year he claimed to have isolated diphtheroids from the cockroach Periplaneta americana. The micro-organisms within the roach were pleomorphic and showed equal and unequal budding. Three morphological types could be distinguished, but they were regarded as strains of the same species. Florence (1924), however, was unable to cultivate the symbiotes of the hog louse; and Gubler (1948) found that in 2000 attempts to isolate micro-organisms symbiotic in the fat-body of Periplaneta americana and Periplaneta orientalis only contaminants were obtained. Again, Toth (1952) described the nitrogen fixing abilities of micro-organisms isolated from aphids, but admitted that he had no reliable evidence that the organisms that fixed nitrogen "in vitro" were those occurring in the aphids. Further doubt was cast on his work by a recent finding suggesting that it is doubtful if aphids need to fix nitrogen as they may well get adequate supplies from the plant sap, (Wigglesworth, 1952). Moreover, Gier (1947) carried out some very careful experiments on the bacteroids of cockroaches. He took fastidious precautions to obtain sterility in his method. He attempted to isolate and culture the bacteroids. His careful investigation of each isolate after each refinement in technique brought him to the conclusion that he had failed to isolate the organism and, moreover,

that he had shown effectively that no previous worker had succeeded.

Thus, one is left with a suspicion that many of the isolations previously claimed were actually, contaminants. However, there are two instances where there seems little doubt that the investigators did indeed isolate the real symbiotes; and for this reason: that they were able to remedy the results of removal of the organisms by re-infection from their laboratory cultures. Brecher and Wigglesworth (1944) isolated an Actinomycete from Rhodnius, and Pant and Fraenkel (1950) isolated yeasts from two beetles. Their investigations are detailed later.

If the micro-organisms associated with insects in special circumstances and in special organs that show no evidence of being parasitic are indeed of benefit to the insect, it is of obvious interest and importance to determine what benefit or benefits they confer upon their hosts. It is not difficult to envisage the very worthwhile benefits that the micro-organisms must receive from the host insect in the form of shelter and food, and perhaps an ensured future for their progeny through hereditary transmission to generation after generation of insects. Caullery (1952), in his book, mentions that Roubaud noticed a strict correlation between haematophagy and the presence of internal micro-organisms that he regarded as symbiotic. Wigglesworth later modified this by pointing out that it was true of those insects whose sole source of nutrition throughout their lives was blood. Thus bedbugs and lice have symbiotes whereas mosquitoes, whose larvae have a more varied diet, are, by report, free of symbiotes. The tse-tse fly, whose larvae are nursed within the mother, a haematophagous fly, harbours symbiotes. It was, apparently recognised that blood was a limited dietary source and it is now

known to be lacking or very deficient in certain vitamins.

It is only recently that our knowledge of the insect micro-organisms and their possible function has attained a secure and satisfactory position within the body of scientific knowledge. This is largely due to the work of Fraenkel and Wigglesworth and their associates. In some work published in 1944 Brecher and Wigglesworth were able to show that an Actinomycete, which they named Actinomyces rhodnii, was essential to the bug Rhodnius prolixus. The insect was obtained free of symbiotes by sterilising the surface of the egg and feeding the young under sterile conditions. Development was seriously retarded but became normal if the bugs were reinfected with the Actinomycete. It is of considerable interest to note that in an earlier paper Wigglesworth (1936) had described the organism as a bacterium of the diphtheroid type and it was, apparently, only as a result of the discovery of Erikson's (1935) report to the Medical Research Council that it was realised that the organism was an Actinomycete. Few, if any, other insect micro-organisms regarded as beneficial to the insect have been classified as Actinomycetes.

Fraenkel, in the last decade, has been making a study of insect nutrition. In the course of this work he and his associates (see Fraenkel 1952) have shown that the vitamin requirements of two species of stored products pest beetles, Lasioderma sericorne and Stegobium paniceum differ conspicuously from those of a number of other insects in that they grow well in the absence of certain individual factors of the B complex which have been proved to be essential for most other insects so far investigated. Lasioderma and Stegobium were known to contain normally mycetomes and associated micro-organisms and it was shown that

removal of micro-organisms was associated in the beetles with a change in nutritional requirements so that they fell in line with those of other insects. A study was made of the importance of each of nine individual vitamins in normal and symbiote free larvae. The results, it is claimed, showed clearly that the symbiotic organisms (said to be yeasts) supplied to the insects significant amounts of thiamin, riboflavin, nicotinic acid, pyrodoxin, pantothenic acid, choline, biotin and pteroylglutamic acid. The yeasts also served as a source of sterols. Recently Pant and Fraenkel (1950) have claimed success in cultivating the yeasts of Lasioderma and Stegobium outside the body of the host. Furthermore, they were able to interchange the yeasts and infect Stegobium with the yeast from Lasioderma and vice versa. The yeasts retained their characteristic shapes and physiological functions in the new hosts. It seemed, however, that the yeast normal to Lasioderma was a better source of vitamin supply than that normal to Stegobium. The Lasioderma yeast, it was claimed contained larger amounts of thiamin, pyridoxin, pantothenic acid, biotin and pteroylglutamic acid. This finding co-ordinates very well with the earlier finding of Blewett and Fraenkel (1944) that Lasioderma grew well in the absence of certain vitamins of the B group that were apparently required by Stegobium.

Some further evidence for the beneficial activities of the symbiotes can be found in the work of deMeillon et al which is quoted by Fraenkel (1952). The experiment was conducted with bed-bugs fed on normal rats and those fed on rats fed a vitamin deficient diet. Where the rat diet was deficient in thiamin the growth rate of the bug was unaffected, but the number of eggs produced was drastically reduced.

Organisms believed to be of benefit to the host insect have been described from Orthoptera, Homoptera, Hemiptera, Anoplura, Coleoptera, Diptera and Hymenoptera. The organisms have been described as rickettsias, bacteria, yeasts, fungi, actinomycetes and protozoa.

Recently, Lanham (1952) offered evidence for regarding the micro-organisms in aphids as merely cell inclusions. This claim may surely be regarded as, at least, dubious in view of the whole body of knowledge outlined above; the recent scholarly work of Buchner (1953); the criticism of Lanham's work by Trager (1952); and the recent finding by Riszki (1954) of desoxyribose nucleic acid in the cockroach micro-organisms.

There is therefore good evidence and some sound scientific tradition for accepting as a premise, at least, the notion that certain of the bacterial like structures existing in insects are living organisms, and that some of these associations between insects and micro-organisms are of benefit to the insect.

(b) The Present Problem

Many beetles have been described as harbouring symbiotic micro-organisms. The weevils or snout beetles are no exception. The granary weevil Sitophilus granarius, L., (also named frequently in the literature, Calandra granaria, L.,) and the rice weevil Sitophilus oryza, L., (also Calandra oryzae, L.,) are both notorious international pests of grain. The two species are closely related. The present investigation was stimulated by statements made in three authoritative texts (Jeannel, 1949; Steinhaus, 1946; Wigglesworth, 1950) to the effect that while Sitophilus oryza always had mycetomal micro-organisms, Sitophilus granarius did not. This statement was derived from Mansour (1935). The implication which

may be drawn from this finding is that one weevil can live successfully without associated micro-organisms or else that the micro-organisms are of no benefit to either host; and are simply of casual occurrence in S. oryza. Clearly, if removal of the micro-organisms from S. oryza caused a serious disturbance in its metabolism it would indicate that this species might need them for proper functioning. Indeed the situation as described stimulated the writer to attempt to distinguish between the two species of weevil by feeding them grain treated with an antibiotic (see below). The two species of weevil did indeed react differently. This finding, however, served temporarily to complicate the whole picture as it was found that in an obscure Egyptian journal Mansour had admitted that it was only a certain strain of S. granarius that was free of organisms. This observation had been overlooked by the three textbook authorities. Personal observation confirmed that S. granarius did in fact contain mycetomal micro-organisms.

In 1930 Mansour had described a "bacterial cell mass" occurring in S. oryza, the rice weevil, and gave a life history of the organisms. The organisms with slight modifications in life history were described in 1933 by Tarsia-in-Curia. Rather similar organisms and a life history were described by Scheinert in 1933 as occurring in S. granarius, the granary weevil. Then in 1935 Mansour in a widely read journal published (1935a) his finding that S. granarius, had no micro-organisms. This would seem to have been the source of the erroneous statements of Jeannell, Steinhaus and Wigglesworth, who remained unaware of Mansour's Egyptian paper (1935b) and its significance until Musgrave and Miller (1953) drew attention to the ambiguity. The German investigators seem however to have been well

aware of Mansour's paper postulating a micro-organism free strain from Egypt and accepted it as the African or Egyptian strain (Koch, 1936).

The position seemed to be that, while S. oryza always had mycetomal micro-organisms, S. granarius could apparently dispense with them since an African variety existed free of micro-organisms.

In progressing towards a full understanding of the relationship between the weevils and their mycetomal micro-organisms, an understanding that may not be achieved for some years, it seemed that a number of lines of enquiry might profitably be pursued.

The object of the present work has, therefore, been to achieve some clarification of the relationship existing between the weevils and their micro-organisms, by studying the distribution of the micro-organisms in the two species of weevils, the morphological appearances of the organisms, and any life history stages that they might exhibit; by attempting to remove organisms from the weevils, in order to discover what, if any, function they may have in the insect economy; by investigating the possible means of transfer of organisms from weevil to weevil; by seeking confirmation of the findings of previous workers; by comparing the biological association in the two weevils; by attempting to cultivate or maintain alive the micro-organism outside the insect body; and by seeking some general clarification of their Systematic position.

(c) Relevant Features of the Weevils' Biology

Both species of weevils live on wheat grain and are reported to be able to feed on a variety of other stored products. Both prefer grain that is not completely dry, and indeed both species have been reared in this work at approximately 27°C and 76% relative humidity.

The species are separated by means of the difference in the shape of the punctures on the elytra and pronotum: they are elongate in granarius and circular in oryza. The species are further distinguished by the fact that oryza usually has four golden-coloured markings on the elytra; moreover, it is, on the whole, the smaller of the two species, and it has true membranous wings under its elytra and it can fly. S. granarius has no membranous wings under the elytra and cannot fly.

A considerable amount of work has been done on the ecology and habits of the two species (see, Cotton, 1920; Back and Cotton, 1924; Ewer, 1945; Reddy, 1950; and Richards, 1947 and 1948). The method of oviposition of the weevils is apparently identical in the two species: the female bores a hole in a grain; deposits an egg in the hole and covers it with a cement plug. Close observation is needed to detect grains containing eggs. The larvae are legless grubs and each spends its whole life inside one grain where it eventually pupates. Finally, as an adult weevil it bores through the wall of the grain and emerges to the outside.

The mycetomal micro-organisms are found, in the larval stage, in special mycetomes. Each larva normally has one mycetome which lies in a U-shaped fashion partly around the oesophagus, with the greater portion of its structure ventral thereto. Each mycetome is a solid

mass of tissue made up of a number of constituent mycetocytes. These mycetocytes contain abundant quantities of micro-organisms. There is no direct communication between the mycetome and the gut. During the metamorphosis of the larva through the pupa to the adult the mycetome breaks down into its constituent mycetocytes. (Murray and Tiegs, 1935). These are carried in a posterior direction into the developing adult midgut. Here they eventually enter the walls of the larger and more anterior midgut coeca as they develop from the midgut. Thence, according to Mansour (1930) and Tarsia-in-Curia (1933) they break into the lumen of the gut. Mansour considered that they were passed to the exterior with the faeces (but see below). In an ageing culture there is usually a considerable amount of frass to be found; yet transmission of the micro-organisms from generation to generation is effected through the gonads (see Mansour, 1930; Murray and Tiegs, 1935; and Tarsia-in-Curia, 1933).

The sexes of the weevils are distinguishable in S. oryza, with practice, by a difference in the length of the snouts or rostra; but in S. granarius no reliable distinction can be made on external characters and dissection of the gonads is necessary (Richards, 1947).

It seems that both species exist in two or more strains separable by weight (Richards, 1944 and 1947; Smith, 1952), though so far no distinct characters exist for separating the strains (Richards).

(d) Brief Review of the Literature Pertaining to the Mycetomal Micro-organisms of the Two Weevils

In his 1930 paper on the "bacterial cell mass" of Calandra oryzae, Mansour referred to his own earlier paper on the embryology of the weevil

and declared that the work of Pierantoni (1927) "seems to be based mainly on my previous work". It seems that Pierantoni referred to this organ as an "organo symbiotico" with no evidence concerning the exact relationship between the micro-organisms contained within the cells of this organ and the weevil in question. Mansour seems to have understood the term symbiotic to mean what is now often called mutualistic. It is uncertain if he was ever convinced that they had any beneficial relationship with the host: there was indeed at that time no evidence that could be regarded as convincing in support of this view. Mansour regarded the organisms as bacteria and found them to be gram positive. His most precise study was made with S. oryza, whose organisms he described as bacilli which occurred singly and in strings. They were longer and more numerous in the egg, larva and pupa, shorter and more segmented in the prepupal and adult stages; the "bacilli" did not pass into the larval gut, but did pass into the cells of the adult midgut. In the adult they showed great activity and "the bacteria in a newly infected gut cell grow actively and form a spherical mass". Eventually the bacteria reached the gut lumen and passed on to the hind gut where they became coccoid and passed out in this form with the faeces, in which rod forms were virtually absent. Infection of a new generation of weevils took place through the infection of the female gonads. Micro-organisms could not be found in the male gonads examined. Mansour at that time considered that the situation was the same in S. granarius. Later (1935a) he declared S. granarius to be free of organisms and hinted that it was therefore unlikely that the micro-organisms were of any significant value to the insects. Soon afterwards, however, (1935b) he stated that it

was only an Egyptian variety that was free of micro-organisms.

Tarsia-in Curia (1933) studied the organisms in S. oryza. Her account was in many respects similar to that of Mansour. She claimed to have observed a reduction in the number of symbiotes during adult life, but considered that this was not so much because they passed to the exterior as that they passed to the female reproductive organs as a result of the rupture of the gut cells. She regarded certain small rods, less than a micron in length, which she observed among the other organisms, as spores and suggested that they were the means of infection of the reproductive organs. She also claimed that the micro-organisms were very active in the adults.

Scheinert (1933) studied the micro-organisms of a number of beetles and his investigations, which seem to have been careful and precise, included S. granarius. His findings can be summarised as follows: the symbiotes are predominantly of the chain form, the chains being longest in the larvae where they reach a length of 40 to 60 microns and a width of 0.7 to 1.1 microns; they are gram positive; in the imaginal midgut they break down into single links 3 to 6 microns long. The freed links were not motile (contrasting with Mansour's finding). The bacteria were present in the eggs recently oviposited. The terminal chambers of the ovarioles contain small bodies that are the results of divisions of single links. The male gonads contained micro-organisms some of which he considered were likely to be transferred to the female weevils.

Murray and Tiegs (1935) stated that they found mycetocytes at the tips of the ovarian tubules. These mycetocytes were similar to

those in the mycetome around the oesophagus. They concluded: "Doubtless their function is to infect the developing ova".

There are thus accounts of organisms with some kind of a life cycle in both species of weevils. The different accounts do not always agree; nor do the descriptions of the organisms entirely coincide with the conventional definitions of bacteria. Perhaps some non-bacterial micro-organism is involved.

II. ANALYSIS OF OBJECTIVES AND METHOD OF APPROACH

It will be seen that from the beginning the problem of the relationship between the Sitophilus weevils and their mycetomal micro-organisms presented certain difficulties peculiar and inherent to itself.

There has been scepticism in some quarters about the existence of micro-organisms. It has been claimed that such structures in other insects are merely "cell particulates" (Lanham, 1953). Further, Mansour's two contradictory statements about the relationship existing between S. granarius and its micro-organisms have complicated the whole literature on the subject. No one seems ever to have confirmed that the variety africana, if it does exist, is free of symbiotes.

The method of oviposition of the weevils makes sterilisation of the eggs without killing them a doubtful practical course of action. There is, of course, no certainty that centrifuging wheat grains would have the effect only of displacing organisms: more serious damage might be caused to the embryo itself. It is not easy to know when a wheat grain contains eggs unless it is soaked in water for a while and obtaining eggs of a known age could clearly prove to be a very long and laborious procedure.

Then there is the great difficulty of culturing the mycetomal micro-organism with certainty. It has already been pointed out that Gier (1936) doubted all previous claims at isolation. Indeed he went further and suggested that the micro-organisms he dealt with had become

such a part of the insect economy that, he thought, with techniques at present available, no isolation could be made. Therefore in this work extreme caution has been adopted in attempting isolations and stress has been laid on special methods rather than repeated unsuccessful attempts with conventional methods. Then, too, it was constantly borne in mind that any isolate would possibly have quite a different appearance "in vitro" from its appearance "in vivo".

If there are strains of weevils that are indeed free of micro-organisms, how do they remain free in the face of the constant possibility of reinfection from (say) the faeces of infected weevils? If there are free strains can they be infected by crossing with infected strains?

Furthermore, the most natural food of the weevils, namely wheat and other cereal grains, is not an easy substrate for dietary tests as it is difficult to alter wheat grains with any reliable knowledge of what one has done. It therefore seemed, from the outset of this investigation that certain methods of approach offered the best, or, in some instances, the only chance of success; while other avenues of approach, even though more conventional, seemed likely to prove unsuccessful. For example, it seemed that little progress would be made in determining satisfactorily what kind of micro-organism was involved until a detailed study of it inside the insect had been made to see if any life cycle that it might undergo could be traced. Again, while it was recognised that it might be possible to rid the weevils of all or most of their micro-organisms by some special treatment, it was realised that a very careful examination of the supposedly freed weevils would be necessary, unless, of course, some alternative means of assessing the presence or

absence of the micro-organisms became available. And, having once freed the weevils, how could they be reinfected? However, if, as seemed more likely as the work progressed, the micro-organisms existed in the weevils in a closed cycle as in other insects such as the body louse (see, e.g. Reiss, 1930), then reinfection would be a very difficult or even an impossible accomplishment; comparable, in some respects, to attempting to infect a vertebrate with malaria without the necessary assistance of a mosquito. Then, too, there was the possibility that certain strains of weevils might be naturally free of micro-organisms because they had never been infected or because their metabolism was antagonistic to the full development of the micro-organisms.

Thus the experimental part of this thesis is presented in four main intergrading parts. The first part details observations on living and stained material designed to clarify the life-history of the organisms and to study their morphology and location. The second part describes the attempts made and the techniques used to try to cultivate the micro-organisms and describes the results achieved. A short third section discusses the possible identification of the micro-organisms. The fourth part deals with experiments that were performed to test the validity of the various hypotheses as to the nature, distribution, transmission and function of the micro-organisms.

III. MATERIAL AND METHODS

Special methods will be described in the accounts of the experiments in which they were used. General methods are detailed here.

(a) The Maintenance of Weevil Cultures

During most of the work, the weevils were bred at 27°C and at 70% relative humidity. These conditions seem near optimum (Ewer, 1945; Reddy, 1950). The temperature was kept constant by placing all cultures in a laboratory incubator. The humidity was kept constant by means of humidors. For example, the standard method of setting up a culture was as follows: about 2 gms grain (not weighed) were put into a small vial measuring about 1⅛ inches in diameter and 2½ inches in height. Weevils were placed in this vial. It was then closed by means of metal gauze mesh held in place by a circumscribing strip of medical adhesive tape. This small vial was then placed in a sixteen ounce ointment jar containing a saturated solution of sodium chloride about ½ inch deep. Because of the phase relationship between the sodium chloride and the air above it in the ointment jar the latter became a reasonably good humidor when its cap was tightly screwed on. Weevils could not be selected as to sex but usually very small specimens were avoided. A new generation of weevils was usually available in 28 to 30 days. Cultures were allowed to continue until they were overcrowded or the wheat grain was eaten. The wheat used throughout most of this investigation was a variety called Cornell No 595 and during most of the work it was oven sterilized

or autoclaved to remove any extraneous insect fauna. The weevils did not breed successfully in rice in the husk nor in maize.

(b) Preparation of Insect Tissue for Examination

To obtain micro-organisms from the weevils they were dissected: a weevil was placed in a drop of water on a glass slide; the gut could then be removed easily under a binocular microscope by separating the insect at the junction of the pronotum and the elytra and then pulling - nearly always the whole mid-gut was extracted in this way. To obtain gonads a similar procedure was adopted, but with the last abdominal segment being pulled away from the rest of the body. Female gonads were by this method readily removed complete. Male gonads were rather more difficult to remove and could rarely be extracted in the complete form.

The dissection of larvae was more difficult because of their soft cushion-like consistency and the location of the mycetome; but by using two mounted needles and viewing the dissection by transmitted light it was found possible with a little practise, to obtain mycetomes from larvae, even from those that were quite small, with a very high degree of success.

(c) Methods of Examination

(i) Phase contrast microscopy

Previous workers (with the exception of Lanham, 1953; see p. in this field of endeavour have all employed classic methods of investigation: the examination of stained smears and microtome sections. No previous investigator has made use of the recently developed technique of phase-contrast microscopy. It seemed that a brief investigation of

the possibilities inherent in this technique for use in the study of insect microbiology would, in itself represent a contribution to our knowledge of this subject. Actually, the method proved very suitable. The apparatus required was simple and easy to manipulate. Material for examination could be prepared quickly and easily and could be observed in the living condition without recourse to staining methods. (this, in itself, favoured the use of the technique, for the microorganisms were, at first, found difficult to stain). The dissected organs of the insect were simply crushed and lacerated on the slide by means of a coverslip.

The slide and coverslip could then be moved over to the compound microscope fitted with a phase contrast attachment and examined in the unfixed, unstained, living condition. The phase contrast apparatus of Cooke, Troughton and Simms was used in conjunction with a compound microscope of the same manufacture. Photomicrography was accomplished by means of the leitz "Ibso" attachment and a Leica camera.

(ii) Method for stained material

At first the organisms proved very difficult to stain as dry smears with any degree of satisfaction, but it was eventually found that if the dry smears were first fixed in Bouin's fixative the microorganisms could be stained very easily. Good results were obtained with Short's modification of Heidenhain's haematoxylin (see Bolles lee,1951) especially when counterstained with eosin. Ziehl's method of carbol fuchsin and methylene blue gave useful results. Other stains tried were Cannon's chlorazol black E and acetic orcein, but these were not as satisfactory as the first two. In addition some material was fixed

in 50/50 mixture of glacial acetic acid and alcohol to effect swelling of the micro-organisms.

(iii) Methods used for sterilising equipment

In only certain aspects of the work was sterility deemed to be required. On such occasions instruments were flamed, glassware was flamed or heat sterilized. Insects were sterilized by immersion in a 1:1,000 solution of Hyamine or, more often by immersion in absolute alcohol, followed by a rinse in sterile distilled water. Agars and nutrient media were usually sterilized by autoclaving at 15 lbs per square inch.

IV. INVESTIGATIONS AND EXPERIMENTS

PART I. Studies of the Micro-organisms Inside the Weevils

(a) Analysis of the association of the micro-organisms and the weevils by means of phase contrast microscopy

(1) Adults, pupae and well-grown larvae. By phase contrast the micro-organisms of the Sitophilus weevils could be seen as distinct rod-shaped bodies of variable length, rounded at each end. Internally they showed a fairly uniform consistency but contained one or two opaque granules. Sometimes, particularly when conditions seemed adverse, there were many small granules. Most commonly, in smears and lacerations the micro-organisms were seen as separate individuals, but sometimes they were seen in pairs and bundles. In the mycetocytes which were observed as large spherical cells containing nuclei, the micro-organisms were seen to be usually in motion, and sometimes seemed so to fill the mycetocyte cell as to block out its nucleus. The isolated organisms never gave any satisfactory evidence of being self motile. Such was the general picture.

In some specimens of weevils, particularly in late pupae or very young adults whose mid-gut coeca had not begun development, micro-organisms could be seen apparently sprouting from several small globular structures. Such phenomena are very similar to the figures given by Peklo (1953) and described by him as "germinating cocci".

It was early noted that the micro-organisms in the two species

of weevils were apparently of different size. Soon, with standard laboratory stocks, it became possible to identify the species of weevil by an examination of its contained micro-organisms. In order to clarify this apparent size difference a series of quantitative measurements was made. The smears made in the usual way were allowed to dry and then examined and the micro-organisms were measured by comparing them against the lengths of the sides of the squares of a small graticule slipped into the eyepiece tube of the microscope. Later this graticule was calibrated by viewing a micro-scale etched on a slide. Many measurements were made in both the species of weevils. It was possible to conclude that the micro-organisms in S. granarius were of predominantly greater length than those of S. oryza. Mean length of some typical organisms from S. granarius selected at random was 10.5 microns with a range of from 3.0 microns to 24 microns and in S. oryza the mean length of some typical micro-organisms was 2.4 microns, with a range of from 1.2 microns to 6.0 microns, (see Figures 7 and 8).

Over a period of time a series of observations was made that pointed to the following conclusions:

One Both species of weevils contained micro-organisms in the mycetomes of the larval stage, and usually organisms of similar morphology in various adult structures. That is, one may reasonably conclude that the micro-organisms in both species carry over from the larva to the adult.

Two In both species of weevils the micro-organisms were nearly always positively detected in the female reproductive organs of the adult.

Three In neither species of weevils were micro-organisms ever positively detected in the male reproductive organs.

Four The micro-organisms were found more frequently in the mid-gut samples of S. oryza than in those of S. granarius; for example in one series of observations there were: positive observations - 31 out of 37 in oryza; 16 out of 24 in granarius.

The micro-organisms of S. granarius were further distinguished by the occurrence among them of bodies that appeared to consist each of one of the organisms wound almost spirally inside a spherical droplet.

Unusual phenomena. Occasionally, in the smears and lacerations of S. granarius there could be seen associated with the standard and normally occurring micro-organisms, a number of small bullet-like micro-organisms, which could be detected clearly only with oil immersion lens and which were about one twentieth the length of the standard organisms, about which the tiny structures often moved in an agitated manner almost giving the impression that they were feeding upon or disrupting their larger "hosts". There was never available any evidence to permit the conclusion that these small objects were spores of the larger organisms, though they may perhaps have been seen by earlier workers and so called. It is, of course, possible that they are a second kind of micro-organism. (See p.30

Some insect specimens of both species were seen to contain strange balloon-, ampoule- or yeast-like structures. These will be referred to later.

(ii) Eggs and young larvae. The detection of eggs in hard ordinary grain was found to be difficult. The hard grains were also

difficult to dissect. However, it was found that if the grains were soaked in water until they were visibly swollen, the little plugs left by the females to cover their oviposition borings could readily be seen. The grains could then be sliced in the appropriate places with a small sharp scalpel.

The first eggs were crushed and examined on February 3rd 1954. It was found that eggs of both species of weevils contained a few organisms, but no division of the organisms seemed to be occurring. In one S. oryza egg a mycetocyte was observed. The next day four more S. oryza eggs were examined: nothing unusual could be seen. A recently hatched larva had many organisms. On February 5th an oryza egg was lacerated but no organisms could be seen. In three very young granarius larvae which were lacerated were seen a few standard organisms but, in addition many spherical bodies.

Drop cultures of a granarius egg and first stage oryza larva were made. These are reported on in the description of the hanging drop cultures. (See Appendix I, II, and III).

(b) Analysis of stained material

Observations of stained material showed the salient features already described for both types of standard micro-organisms. It was, however, from observations of this material that it was possible to detect "spore germination and spore formation" of the organisms.

"Spore germination" could be seen as outgrowths of filamentous strands from globular bodies. Sometimes three or four filamentous strands could be seen originating from one spherical body.

"Spore formation" was seen to occur as the breaking off of

the tips of the standard organisms in the form of globules. This is shown in Figure 4.

It was, however, impossible to determine fully the growth cycle of the organisms. No evidence was available to determine if the spores formed in the manner described were the same as those seen sprouting filaments. Perhaps the two phenomena form separate parts of the life history of the same organism. (But see observations in Appendix III).

(c) Weevils apparently free of micro-organisms

After the laboratory stock of weevils had been subjected to as full an investigation as seemed possible, additional insect material was obtained from a nearby laboratory for comparison. Two stocks of weevils were obtained from Mr. H. A. U. Monro, of the Science Service Laboratory, London, Canada. They are referred to in the present work as MW and LG, while the original stock, with which much of the work of this thesis was done, is referred to as the GG strain.

The London strains proved of great interest since they were found to be entirely or almost entirely free of the mycetomal micro-organisms characteristic of the GG strain. The method of examination used in arriving at this conclusion was similar to that already described, for use with the phase-contrast technique. The gonads of female and the midguts of male and female weevils were examined using an 8 mm objective (x20) and a x8 ocular. Under these circumstances the standard mycetomal micro-organisms could be readily seen and could not be overlooked, unless of rare and isolated occurrence. A careful search of several fields was made before classifying any preparation as negative.

Positives were immediately apparent. Examinations of individuals of these two strains were made over a period of several weeks. In this survey, in the MW strain, of 33 insects examined in various stadia only two were found to be positive for mycetomal micro-organisms and one was indeterminate; and in the LG strain, of the 48 examined in various stadia only two were positive. Later observations and examination of material used in later experimental work added abundant confirmation.

In addition to the differences of micro-flora the strains of weevils seemed to differ in other respects. For example, on the whole, individuals of the MW strain were smaller and lighter in colour than those of the GG strain. (But see later, on p. 49).

Individual weevils of the two London strains were sometimes found to harbour the small bullet-like organisms previously mentioned (p. 27) and other anomalous micro-organisms. Moreover females of all strains were occasionally found to harbour a few micro-organisms of unknown origin and affinity in the spermathecal glands and some individuals of the MW strain had small bacilli in hind-gut invaginations. These micro-organisms have not been investigated in the present work.

PART 2. Studies of the Micro-organisms "in vitro"

During the entire course of the work repeated attempts have been made to grow the micro-organisms in some kind of medium outside the insect.

(a) Investigation of cultural methods and techniques

(i) Tissues containing the micro-organisms were dissected out under sterile conditions and lacerated on sterile slides. From such lacerations dilution series were obtained in the following way.

A series of four sterile slides each containing one drop of sterile distilled water was prepared. Then a drop of material from a laceration was taken and transferred to the first of the series of slides and mixed. Material was then taken from this first drop of the series and small quantities of it were allowed to form drops on a petri dish containing agar and held on a slant so that each small drop of the transferred material made a little vertical rivulet on the surface of the agar. Four such rivulets were made on the surface. The lid was then replaced on the petri dish. The material left on the first slide of the series was then used to supply a drop to the next slide in the series; thence the procedure was repeated. Any growth that occurred on the agars could be sub-cultured.

Various culture media were used but no certain isolation of the micro-organisms sought was made. In view of the work of Cier already quoted this is not really surprising.

(ii) Whole mycetomes were implanted, under sterile conditions

on to sterile agar nutrients. Various growths resulted. One, which at one time seemed to be the micro-organisms of the mycetome was of a yellow colour and produced an oily surface growth and was sub-cultured several times and examined microscopically by the phase microscope. It never gave any satisfactory evidence of being the organism from the mycetomes and so was eventually relegated to the classification of "contaminant".

(iii) A method that eventually achieved some success was as follows. Mycetomes, or other micro-organism-containing parts were dissected out on sterile slides from surface sterilised insects. The tissue was then transferred to a drop of sterile water on a sterile coverslip. This coverslip was then inverted on to some sterile agar in a sterile petri dish. It was thought that in this way, if any growth did actually arise from the mycetomal micro-organisms, it would be possible to detect it and be confidant of its source. In order to see the micro-organisms it became necessary to invert the coverslip on to a sterile slide containing a sterile agar. In this way it was possible to observe some sporulation of the micro-organisms. (See Appendix II).

(iv) Hanging drops and tissue cultures

After repeated efforts to isolate the micro-organisms successfully had failed, it seemed that perhaps the only possible method was to attempt to grow the mycetomal tissue of the insect "in vitro" and to cultivate the micro-organisms on it. A long series of slide cultures was made. It was found that it was possible to keep the micro-organisms alive in slide cultures under certain circumstances for nearly a year. However, it was very difficult to be sure that any reproduction occurred.

It was not found possible to grow insects' tissues "in vitro". Goodchild (1954) and Grace (1954) experienced the same difficulty. A list of the slide cultures made is given in Appendix I and III on page 94. It can be seen that the most satisfactory media were two obtained from Dr. R. L. Parker of the Connaught Laboratories in Toronto. The constituents of these two synthetic media are given in Table XIV and further information is available in Parker (1950) and Healy et al. (1954).

Three chief kinds of sterile cultures were made.

Type A The deep-celled slide. These were made in standard hanging drop slides. The cavity was circular and 15 mm in diameter and 3 mm deep. The slides were of standard length and breadth but were 4 mm deep. The upper surface was ground. These slides were flame sterilized and then placed in a sterile petri dish. Just prior to use the cell of a slide would be provided with one or two drops of sterile distilled water. The lid of the containing petri dish was then replaced to cover the slide temporarily. An insect specimen was meanwhile surface sterilized. It was then dissected in sterile distilled water on a sterile slide in air. The mycetome, gut or other desired organ was then transferred from the drop to a drop of sterile synthetic medium on a sterile coverslip. The rim of the cell in the celled slide had meanwhile been smeared with sterile vaseline. The coverslip containing the piece of tissue containing the micro-organisms was then picked up and quickly inverted over the cell in the celled slide. The tissue was thereby retained in the drop of synthetic medium in the cell sealed off from external contamination. The sterile water in the pit of the cell served to keep up the humidity of the atmosphere in the cell. This had been found to be important.

These hanging drop cultures worked extremely well and there were rarely any contaminants in them. They could be readily observed by phase contrast. Their disadvantages were that on several occasions the drops were too deep for the focus of the microscope objective so that only organisms in the margin of the drop could be examined, particularly with the higher powers of the microscope. Moreover, it seemed likely that the phase contrast apparatus was not working to best effect through the thick glass of the celled slide. More satisfactory results were obtained with type C.

Type B This kind of arrangement was devised to test the idea that tissue from some other part of the insect might be affecting the part containing the micro-organisms. Was it possible, for example, that the stimulus causing the initiation of metamorphosis and therefore of the disruption of the mycetome was also causing the micro-organisms to reproduce? This hypothesis was tested by making a double hanging drop. The method was as for type A except that on the sterile coverslip were placed two separate drops of sterile medium each containing one of the two pieces of tissue. A piece of flamed nichrome wire was meanwhile placed in the vaseline at the rim of the celled slide in such a way as to project from the slide a short distance and, at the same time, to project into the cell of the slide to about its center. The coverslip with its two drops was then inverted over the cell of the slide so that the wire lay between the two drops. In this way it was possible at some convenient time later to mix the contents of the two drops by gently oscillating the wire and then sharply removing it by pulling it out through the sterile vaseline and pressing down the coverslip. See Figure 3.

Type C This was essentially the same method as type B except that ordinary thin type microscope slides were used instead of the celled slide. Here the drop of synthetic medium containing the tissue was usually placed on the slide. Sometimes such a drop was ringed with vaseline before the placing of the coverslip but eventually it was found better to apply the coverslip to the drop and then form the vaseline seal by applying the vaseline and causing it to flow by warming. These preparations had the advantage that they were not too thick for focussing or for the phase contrast technique. On several occasions it was found possible to transfer such slide cultures from one slide to another. It should be emphasised that contaminants were rarely seen in these cultures.

These hanging drop cultures proved invaluable in the investigation. Micro-organisms could be maintained alive (or in a life-like condition) in them for many months.

(b) Summary of findings

The results of these observations on hanging drop and slide cultures may be summarised as follows.

The micro-organisms of S. oryza and S. granarius can be maintained apparently alive in hanging drops and slide cultures containing natural and synthetic media. In some instances they have been kept alive for many months. The best media of those tried were Earle's solution and Parker's No 199. (See Table XIV).

It is likely but not definitely established that some reproduction of the micro-organisms took place in these cultures, as evidenced by sporulation and coccoid bodies. (See Figure 4).

No evidence was obtained that nerve tissue from pupae or prepupae

or larvae stimulated reproduction of the micro-organisms.

Micro-organisms from S. oryza and from S. granarius were not found to cause changes in one another's morphology when kept in the same drop.

The presence of the antibiotics, Terramycin and Penicillin in the hanging drop and similar cultures did not appear to be harmful to the mycetomal micro-organisms. On the contrary, they seemed almost to have a stimulatory effect. A chronological list of Hanging drop cultures is given in Appendix I, and an account of one particularly good drop, in Appendix III.

PART 3. On the Possible Identity of the Mycetomal Micro-organisms

The mycetomal micro-organisms seemed to possess characteristics that clearly distinguished them from the true fungi and also from the true bacteria. The following features are worthy of note in this respect:

(a) There is strong evidence that the organisms exist in different forms suggesting that there is some sort of life cycle.

(b) They are too small to be fungi.

(c) The "mycelium" appears to be unicellular.

(d) Long and short hyphal forms occur.

(e) Spores stain more intensely than the hyphae.

(f) "Spores" often germinate by more than one germ-tube.

(g) Peculiar lateral branching has occasionally been seen.

(h) In old cultures certain terminal swellings of the hyphae may be observed. These also occur under abnormal conditions of growth.

(i) Sporulation may apparently occur by segmentation or by fragmentation.

For the above reasons it was tentatively decided that the organisms could be regarded as Actinomycetes. In accordance with this notion the following description of the genus Nocardia is not without significance; it is from Waksman (1950) page 24:

"Slender filaments or rods, frequently swollen and occasionally branched, forming mycelium which after reaching a certain size may give the appearance of bacterial growths. Shorter rods and coccoid forms are found in older cultures." ..... "Paraffin, phenol and m-cresol are frequently sources of energy" ..... "The colonies are similar in gross appearance to those of the genus Mycobacterium." and

"In older cultures of Nocardia many coccoid cells are changed into durable forms. The latter are larger than the vegetative coccoid cells and the plasma of these cells is thicker than the plasma of the vegetative cells. On fresh media the so-called durable cells germinate

like the spores of Streptomyces; they form 2-3 germ tubes. Besides the cells mentioned, numerous involution forms can often be found in older cultures of Nocardia. These cells are thin regularly cylindrical or coccoid and are often transformed into a series of spheroid or elliptical ampoules and club-like forms (2 to more than 3 microns)."

and, finally: -

"Many Actinomycetes produce no aerial mycelium; growth appears to be similar to that of pleomorphic bacteria, like members of the genus Corynebacterium."

Many parts of the above description certainly tally well with observation made on the organisms in S. granarius and S. oryza. Moreover, it is of interest to recall the presence of phenols in insect blood (Wigglesworth, 1930). The isolation of the Actinomycete by Brecher and Wigglesworth (1944) which at first was thought to be a diphtheroid bacillus is surely of added significance. Moreover the Actinomycetes are notoriously pleomorphic; they are difficult to classify and (vide Erikson, 1935) often prove difficult to culture on artificial media, where, furthermore, they grow slowly.

PART 4. Analysis of the Problem by Experimentation

(a) Introduction

The weevils Sitophilus granarius and Sitophilus oryza thus seem to have associated with them certain mycetomal micro-organisms that are probably Actinomycetes. These organisms seem to be to some extent specific, for the species of weevil can be identified by reference to the kind and quantity of its mycetomal micro-organisms. If the micro-organisms do supply the weevils with any dietary constituent it seems that S. granarius is able, under normal circumstances, to obtain this constituent independently as it exists in strains that have few or no mycetomal

micro-organisms, clearly recognisable as such.

Experiments designed to see if S. granarius can be artificially deprived of its micro-organisms, if S. oryza can live without its micro-organisms, if the organisms are transferred congenitally or orally, if they are sensitive to antibiotics and if they are of benefit to the weevils clearly have considerable value in an investigation such as this.

Experiments of this kind are described in the following pages. Where S. granarius is mentioned, the G.G. strain is referred to unless otherwise indicated.

(b) Effect of the antibiotic, Terramycin, on the weevils

The original idea underlying this experiment was the assumption that if S. granarius did not have micro-organisms it would be affected differently from S. oryza by treatment with an antibiotic, particularly, if the organisms in S. oryza were supplying a dietary deficiency to their host.

Experiment 1

Wheat grains were sorted as to approximate size in accordance with Ewer's (1945) indication that in experiments with S. granarius in which uniform oviposition was desired, wheat grains of uniform size should be provided. The grains were weighed as 2 gm samples into small glass vials and treated with acidified solutions of Terramycin hydrochloride (Regna and Solomons, 1950; and Weyer, 1950). Acidification was found to be necessary to avoid the iso-electric point and consequent precipitation of the Terramycin in the solution. Half the total number of vials received 2 ml acidified Terramycin; the other half, the checks received

2 ml acidified distilled water. After soaking, the grain in the vials was dried in an oven at 40° to 50°C. From stock cultures of the two weevils replicate vials of grain were supplied each with 20 weevils of one species. As Richards (1948) had found two physiological races of different sizes in S. oryza and S. granarius (1947), it was regarded as desirable to select the largest available specimens of each species. There were twelve vials: two species of weevils each receiving two different treatments; each treatment in triplicate. The arrangement of the experiment can be deduced from Table I. Each vial was placed in a separate humidor in an incubator. So that conditions approached as nearly as possible to 26-27°C and 76% relative humidity, the tops of the vials were covered with bolting silk held in place by Johnson's medical adhesive tape.

After 72 days a count of weevils was made. The results are given in Table I. During the experiment some S. oryza did escape from two check vials and drown in the salt solution. They were included in the population counts as live weevils; and it may well be that their escape had a lowering effect on the counts of weevils in these check vials (for they might have been females) thus falsely decreasing the difference between check and treated populations, thus making the results less dramatic. Both species of Sitophilus feign death; behaviour that complicates mortality counts. But by the simple procedure of subjecting doubtful insects to the light and heat of a bench lamp some fairly accurate estimate of true mortality could be obtained; and these figures are given in the Table. A second count was made about 40 days later and confirmed the trend shown in the first.

Experiment 2.

A second experiment of more elaborate design was performed. It was arranged so that each of the two species of weevils received three doses of Terramycin: - 0 gm, 10 gm, and 100 gm per ton, in three replicates.

The grains were sorted as in the first experiment and placed as 2 gm lots into small vials where they were soaked in that concentration and volume of acidulated Terramycin solution previously calculated to give the desired dose. Grains that received zero dose of Terramycin were soaked in an equivalent volume of acidulated distilled water. When the grains had thoroughly soaked up the solutions rapid evaporation was encouraged to prevent decay or germination of the wheat grains.

There were eighteen small vials. They were placed in six ointment jar humidors containing saturated solutions of sodium chloride.

Details were as follows:

0.1 gm.of Terramycin hydrochloride was dissolved in 100 ml of distilled water acidulated with 0.5 ml of N HCl to prevent precipitation of the Terramycin. This solution was poured on the grain in six vials at the rate of 2 ml per vial. The solution was then diluted 1 to 100, and added to another six vials. The remaining six vials received 2 ml of acidulated distilled water of pH appx. = 2 (0.5 ml N HCl to 100 ml water).

Each vial was subsequently supplied with 20 to 21 weevils of either granarius or oryza. The following code was used: G = granarius; O = oryza; D = distilled water; S_1 = high dose Terramycin; S_2 = low dose of Terramycin. The vials were covered with bolting silk as in the previous experiment and they were then placed in humidors as follows:

Humidor 1: - vials GD GS_1 GS_2.
Humidor 2: - vials replicates of above.
Humidor 3: - vials replicates of above.

Humidor 4: - vials OD OS_1 OS_2.
Humidor 5: - vials replicates of above.
Humidor 6: - vials replicates of above.

There were thus 60 insects in each humidor and 60 insects given each kind of treatment.

The results are shown in Table II.

In considering these results it may be noted that drowned insects do not appear to be associated with any unexpected variation in population. Moreover, it is to be remembered that the precise number of insects per vial was open to a slight variation: then, too, the sex ratio in each vial was undetermined. The results may certainly be regarded as showing a trend.

These experiments were performed before it was appreciated that S. granarius frequently had mycetomal micro-organisms. They were, in fact, performed on the assumption that Mansour's statement (1935a) about the absence of these organisms in granarius was correct. There became little doubt that the stock of S. granarius used in the experiments did, in fact, contain mycetomal micro-organisms (see, for example, the next series of experiments). Therefore, the question which presents itself is, was the differential effect of the Terramycin due to an indirect effect of the antibiotic on the insect, caused by removing its essential associated micro-organisms; or was it due to a direct toxic action of the antibiotic. In the former instance, one would naturally assume that S. granarius can survive without its micro-organisms, whereas S. oryza

is more dependent upon them. It later became apparent that S. granarius can, indeed, survive without its organisms for the M.W. and L.G. strains were apparently free of them. It seemed desirable, nonetheless, to perform an experiment directed to determining the effect of the Terramycin upon the micro-organisms in the two species of weevils.

(c) The apparent effect of the antibiotic Terramycin on the mycetomal micro-organisms of the two species of weevils

The object of these experiments was to treat grain with Terramycin, feed it to the weevils and then examine the weevils for micro-organisms, using weevils fed water-treated grain in the checks. In this way it was hoped to see if the Terramycin did indeed affect the micro-organisms.

Experiment I.

The method was as follows. Small glass vials containing 1 gm of grain each were used as in the above experiments to treat the grain with the Terramycin or the acidulated water. The solutions were as follows: Terramycin hydrochloride: 1 gm Terramycin dissolved in about 15 ml of distilled water, 2 ml of N HCl were added and the whole made up to 20 ml with distilled water. The acidulated water was made up by adding 2 ml of the N HCl solution to 18 ml of distilled water. The solutions were applied to the wheat grains by means of a manually operated micro-syringe, at the rate of 1 ml of solution per gm of grain. The vials were then tilted to encourage soaking of the Terramycin by the grain. When the Terramycin had been fully soaked up, the grains were rapidly dried. Each vial was then placed in a separate humidor with a salt solution as in the previous experiment. The vials were supplied

wire gauze coverings held in place by medical adhesive tape. Each of four of the vials was then supplied with 25 weevils and the humidors, with vials, were placed in the incubator at 27°C.

Summarizing:

1 vial contained water-treated wheat and 25 granarius.
1 vial contained water-treated wheat and 25 oryza.
1 vial contained Terramycin wheat and 25 granarius.
1 vial contained Terramycin wheat and 25 oryza.

All the oryza were from one culture; all the granarius were from one culture.

The experiment was set up on 16th of June, 1952.

The weevils in the various cultures were examined at intervals for micro-organisms as shown in the table (Table III).

The results indicate that, at first, the Terramycin had no effect on the micro-organisms; but that after a few weeks the treated weevils were negative and the untreated, positive.

Experiment 2

Table IV shows the results of a similar experiment in which the wheat was treated at the same time as in the immediately preceding experiment, but the weevils were added on July 5th, 1952.

Table V gives the condition of the cultures with regard to their total populations produced within the duration of the experiments. These figures show a remarkable similarity to those given by the experiment shown in Tables I and II in that they indicate that S. granarius is less affected by the treatment than S. oryza. The numbers of the treated oryza

might possibly have been affected by the search for larvae during the course of the experiment, but this seems doubtful for if there had been many larvae and therefore potential adults, so prodigious a search for larvae would have been unnecessary.

(d) An experiment to ascertain if the Terramycin ingested by the weevils is detectable within them

The object in this experiment was to treat weevils with Terramycin and then see if the alimentary canals of such treated weevils inhibited the growth of organisms susceptible to Terramycin.

The method was as follows. Two gm quantities of sterilized wheat grains were weighed into each of six small glass vials in which the grain was treated. Three of the vials were treated with a solution of Terramycin hydrochloride (0.5 gm of Terramycin in 40 ml of acidulated distilled water) at the rate of three ml solution per vial. The other three vials were treated with acidulated distilled water at the rate of three ml per vial. These were the checks.

The vials were supplied with weevils.

About a month later cultures of Escherichia coli were prepared on Bacto nutrient agar in petri plates. Some weevils were then removed from the vials, washed in sterile distilled water and dissected and the mesentera and gonads placed on the agar. Then as a check for the possibility of external contamination of the weevils a whole adult was removed from a Terramycin culture and dropped on to the centre of the agar plate. Evidences of inhibition were sought 24 hours later. The only zone of inhibition that could be discerned was around the whole adult in the centre of the plate.

This result suggests on first analysis that the weevils are not ingesting the Terramycin. This, however, is very unlikely since they had eaten the grain. Also it was likely that they were still eating the treated grains at the time of the dissection. Then it seems either that the Terramycin present in the mesenteron and gonads was not passing out onto the agar or else that it was not in sufficient quantity, in the weevils dissected, to affect the E. coli organisms.

(e) To determine if the mycetomal micro-organisms are absent from old weevils

Mansour (1935) seemed to think that as an individual weevil increased in age the number of its micro-organisms decreased until there were none. If such were the case it would clearly affect the results of observations in some experiments and the results of observations sometimes made on stock cultures. It seemed, therefore, desirable to determine if indeed it were so.

One culture of S. granarius was selected and all weevils were removed from it at intervals and some of these were placed in small vials with grain. These subsidiary cultures were set up in humidors in the usual fashion. At intervals of thirty days or less these subsidiary cultures were opened for examination. At this time the grain was replaced with fresh grain so as to prevent second generation weevils emerging into the cultures and thus nullifying the whole experiment.

The results of this experiment are shown in Table VI. It can be seen that there is no evidence that the micro-organisms are absent from older weevils.

(f) Effect of temperature on the micro-organisms of the two species of weevils.

As Buchner has suggested that the so-called Egyptian variety of S. granarius became free of micro-organisms as a result of exposure to high temperature, it seemed desirable to determine what effect temperature would have.

The two species of weevils were reared at three different temperatures at constant humidity. Three standard humidors were used; each contained one granarius culture (grain and 12 adult weevils) and one oryza culture (grain and 14 adult weevils).

The humidors were placed in incubators running at 20°C, 26-27°C and 30°C. The temperature of 30°C was chosen as previous observation indicated that 35°C was too high and an incubator running at 30°C was available. The experiment was set up on May 18th, 1954. The temperature in the 26°C incubator fluctuated very slightly: about ± 1°C. But the other two incubators had a greater fluctuation.

The first examination of the cultures was made on 4th of August, 1954, after they had had good time in which to build up; and examinations were made at intervals thereafter. The S. oryza reared at 27°C was a poor culture for no apparent reason.

In the male weevils only the mid gut was examined: in the female weevils, both mid gut and gonads were examined. The results were as follows:

At 30°C :	S. granarius,	20 positive; 12 negative
	S. oryza,	8 positive; 3 negative
At 26°C :	S. granarius,	7 positive; 0 negative
	S. oryza,	1 positive; 0 negative

At 20°C : *S. granarius*, 7 positive; 0 negative
S. oryza, 9 positive; 2* negative

(g) The effect of tetrazolium on the mycetomal micro-organisms

The compound, 2,3,5-triphenyl tetrazolium chloride was apparently first developed by Lakon (1949) as a vital stain for detecting living tissues. An excellent account of its uses is to be found in the review by Smith (1951). It is one of the few organic compounds that is coloured in the reduced state. It has been found to turn pink in the presence of certain actively growing tissues.

Tests were carried out with compound but the results were not really satisfactory. The solution was made by dissolving 0.068 gm of the tetrazolium salt in 10 ml of distilled water.

It was found that oenocytes stained pink very rapidly and then eventually went purple. Salivary glands also took up the stain. The mycetomes and micro-organisms, however, did not take up the stain so well. Mycetomes stained slowly and usually the pink stain in the micro-organisms was seen only under phase or dark field. The micro-organisms stained best when immobile and apparently dead.

As a check on these unsatisfactory observations the tetrazolium was tested on a culture of *Bacillus subtilis*. A general pink colouration was seen after some time; but this pink colouration could be seen in the individual bacilli clearly only with the aid of the phase. That is, their staining reactions appeared to be essentially similar to those of the

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* Because of dissection failure, one of these was from only a part of the gut of a male; the other from the gonads of a female.

mycetomal micro-organisms. A similar poor effect was obtained with the tetrazolium on Aerobacter sp.

It seems that the poor staining of the mycetomal micro-organisms is of no special significance.

(h) The effect of strong salt solution on the micro-organisms

In order to see if the micro-organisms gave any characteristic reactions some tests were made with a strong solution of NaCl. A 25% solution was used.

Observations By phase.

On S. oryza organisms: vacuoles developed, giving some of the organisms a beaded appearance. Others showed a marked granular appearance. Still others seemed unaffected.

S. granarius organisms: in the salt solution the organisms could be seen "writhing" and making sudden movements; others became swollen in the middle, others at one end; others showed separation marks; many developed vacuoles. It seemed that none disappeared.

(j) Weights of strains of S. granarius

It seemed from observation that the MW strain of weevils mentioned on p.29 was, on the whole, comprised of individuals smaller and lighter in colour than the individuals of the CC strain. Weevils of the LC strain seemed mostly lighter in colour but only slightly smaller in size. Koch (1936) quoted observations of Zacher who claimed the existence of an Egyptian or African strain of S. granarius which he named var. africana.

Koch implied that this variety was the same as Mansour's micro-organism-free free strain from Egypt; and that it was a strain adapted to warm temperatures. Buchner accepted this theory. Richards (1948) after some very careful and extensive observations found that the weights of weevils were dependent upon factors of age, nutrition, individual rate of development, stage of the culture in which the adult emerged and general environmental conditions. Nonetheless, he was able to establish the existence of three genetic weight strains; when reared in 3x1 inch vials in his laboratory these had mean weights of 1.90, 2.07 and 2.46 mgm. It is clear, however, that care and caution must be exercised in claiming size differences in the weevils.

Nonetheless some weevils in the three strains available were weighed. The results are given in Tables VII and VIII. The weighings were made on a Sartorius-Werke, damped, two-pan balance and the weevils were weighed in batches of three or more, as the balance seemed insufficiently accurate to weigh single weevils. Weevils were selected at random, and specimens from different strains were weighed at the same session.

The observations have been frequently born out since these readings were taken and the general conclusion has therefore been that the GG strain is definitely heavier and darker than the other two strains. Whether these two latter strains really differ significantly is not certain.

(k) The transfer of micro-organisms from one generation of weevil to another

According to previous investigators the micro-organisms in the weevils are transmitted from generation to generation of the weevils. Mansour and Tarsia-in-Curia considered that this transfer took place through the female weevils only, whereas Scheinert and Buchner considered that it took place through both males and females.

The two strains MW and LG, from the London Laboratory, which apparently represented strains of Sitophilus granarius that were free of mycetomal micro-organisms seemed therefore to supply excellent material for the investigation of this matter. It was therefore decided to attempt to cross the stock GG strain and the strains from London. This proved to be more difficult than was thought likely; for the great difficulty in sexing S. granarius became a source of trouble and delay.

The following method was eventually adopted, with some degree of success.

Individual grains from cultures of the respective strains were sorted into observation containers. Some of these containers held several grains; some held only one grain. The object was to obtain young adult virgin males or females. The weevils from the MW and LG strains were marked with a small spot of white paint on the thorax. Pairs comprising one partner from strains MW or LG and one from strain GG were then placed together and frequent observations were made to see if copulation took place. If it did not occur within 24 or 48 hours the individuals were confined with other partners. Notes were kept of the transfers and successful pairings.

Details are given in summarized form below.

16 June 1954 The three strains were check examined:

LG negative
MW negative
GG positive

18 June 1954 Vials numbers 1 and 2 each supplied with two weevils; one in each from the GG strain and one in each from the MW strain.

19 June 1954 Pairing occurred in vial number 1, and it was apparent that the female was of MW stock. On the 21st June this female was transferred to a standard culture vial containing grain and held in a standard humidor. This female was the progenitor of Culture ex 1.

25 June 1954 Mating was seen in vial 2. The female was MW. This became culture ex 2.

24 June 1954 Pairing occurred in vial 4 where the female was again seen to be MW. This female was the progenitor of Culture Ex 4.

Vial 5 failed as both weevils died.

29 June 1954 Two grains of wheat were removed from the same observation vial of GG strain weevils. The grains were opened up and two weevils removed. They were both obviously mature adults and it was assumed that they were virgins. Both were tentatively sexed as females. They were put in separate vials: numbers 6 and 7. A supposed male of the LG strain was introduced to each of the females (?) in turn. Mating occurred in vial number 6 and it was clear

that the dotted specimen (LG strain) was the male. The female of this strain was the progenitor of Culture ex 6.

A ♂ male from MW was placed in vial 7 with the GG supposed female.

8 July 1954 The two specimens from vial 7 were examined microscopically. Both proved to be females; both gave evidence of being virgin.

Another supposed pair was introduced. Mating occurred and the female was MW. She became the progenitor of the Culture ex 8.

About one month later careful microscopical examination of the offspring of these pairs began and extended over a week or more. In the course of these examinations one particularly interesting observation was made. In Culture ex 6 was found the original father of the culture still with the white dot on him. A laceration of the gut and part of the gonads showed no standard micro-organisms. That is, he was true to his strain.

In examining these weevils it was realised that it would inevitably take longer to decide about the negative results than about the positive. One quick glance usually revealed a positive specimen - the micro-organisms were present in tens of thousands. A specimen could be classed as negative only after a conscientious search had been made. It was decided that if five or less certainly identifiable standard micro-organisms could be seen the specimen was negative. This decision was arbitrary but seemed justifiable as it was found that there were very few borderline cases. The results were as follows:

Culture ex 1 MW ♀ x GG ♂
50 weevils examined: 1+, 3?, 46-.
Examinations made up to 1st Oct.

Culture ex 2 MW x GG
Only two weevils were found;
both were females: 1+, 1-.
Obviously these were the original
supposed pair.
Terminated 1 Oct.

Culture ex 4 MW ♀ x GG ♂
13 weevils examined: 13-.

Culture ex 5 Both weevils died early

Culture ex 6 LG ♂ x GG ♀
43 weevils examined: 42+;
and the original male: negative.
Examinations made up to 1st Oct.

It is claimed that these results offer a very striking proof that the weevils inherit the micro-organisms only through the female.

The weevils in culture ex 1 were typical of the MW strain: pale chestnut colour and of "slight" build. Those in culture ex 6 were typical GG strain: dark mahogony colour and "heavy" build. The difference in appearance was very striking. Indeed, it was as a result of these observations that the writer was led to make a careful study of size and colour differences of the three strains. One further point, the homogeneity of the populations was also striking. This, in conjunction with other observations, has led to the suspicion that the MW strain as a whole is very homogeneous, whereas the GG strain (except those in cult ex 6 derived from one female) is not completely so. These two strains, however, were readily distinguished when reared under the standard near optimum conditions.

1) To investigate possible differences in the nutritional requirements of the strains of S. granarius

Clearly, if the mycetomal micro-organisms do really confer benefit upon their hosts by supplying them with needed vitamins of the B complex, the strain of weevils that normally harbour micro-organisms should be more adaptable to changes in the nutritional value of the grain than the strain that normally does not harbour them. Is the MW strain, for example, less well adapted in this respect than the GG strain?

As the weevils seemed to prefer to oviposit in an ovoid-shaped body and as the natural habitat for the larvae is within a wheat kernel it seemed that studies in nutritional differences would best be accomplished by attempting to alter the chemical nature of the wheat kernels rather than by trying to rear weevils in an unnatural physical environment such as a powdered synthetic food.

Enquiries and investigations indicated that perhaps the most effective way to alter the wheat grain chemically without altering them too drastically physically would be to "pearl" them. This pearling process is the same as the pearling process in barley; it removes the greater part of the outer coat of the kernel as well as the embryo and makes the ovoid wheat grain more spherical. Of course, by this method it is impossible to be certain what vitamins had been removed, but enquiry from grain specialists revealed that it would be impossible to be certain what had been removed from wheat grains subjected to any particular kind of treatment.

Some experiments were therefore performed using the pearled wheat as a deficient dietary source.

The pearling was done in a small pearling machine in the Field Husbandry Dept. O. A. C. It was used chiefly to pearl barley.

First experiment

The standard Cornell wheat was pearled for two minutes and 2.5 gm quantities of it were weighed into the standard small vials. 2.5 gm quantities of ordinary unpearled wheat grains were placed in other small vials. Then into each vial were placed seven weevils: as nearly as possible, six females and one male. The vials were placed in humidors in the usual way and placed in the 27° incubator for cultures to be produced in the vials. Details are given in Table IX.

Second experiment

Four of the standard vials were supplied with 2 gm of wheat each. Two vials received pearled wheat, two unpearled. Twenty weevils were placed in each vial; and each vial was placed in a humidor in the usual way. Observations were made and recorded. After the experiment had been set up for a few days all the weevils were removed from the cultures. The removed weevils were sexed by dissecting them. Their removal, it was thought, would prevent the oviposition of a greater number of eggs than the grain could support, and would also prevent weevils of the progeny emerging over a prolonged period of time. At the end of the experiment the total number of weevils in each culture was weighed and the mean weight of weevil per culture thereby obtained. Details are given in Table X.

Third experiment

If pearled wheat is a deficient dietary source, this may be due to certain of the B vitamins having been removed by the pearling process.

Then if pearled wheat is treated with certain B vitamins does it become once more a satisfactory dietary source? This experiment was designed to see if any trend in this direction could be detected.

4 gm samples of wheat were weighed into the standard vials. Some of these samples were then treated with a vitamin solution and one with a check solution. The solutions were then evaporated off. Cultures were then set up using these wheat samples.

The application of the solutions was so arranged that the grain, in theory, received 4mgm/gm of each of the following B vitamins: niacin, thiamine, calcium pantothenate, riboflavin. As this was an exploratory experiment all figures were approximate. Moreover, the dose of vitamins was very high. In some of his work, Fraenkel had used some of the same vitamins at a dose of 500 microgrammes/gramme of food material.

It should be emphasised that this experiment was regarded as a simple exploratory test; for it is well appreciated that the design and performance of nutritional experiments has in recent years attained considerable precision and complexity. Details of the experiment are given in Table XI.

Fourth experiment

Details are given in Table XII.

Fifth experiment

This was a more elaborately designed experiment. The objects were to determine whether the strains MW and CC were differently affected by being fed pearled wheat, and to what extent treating the pearled wheat with constituents of the vitamin B complex made it a satisfactory dietary source. Pearled wheat was divided into three samples. One was left

dry; one was soaked in distilled water; one was soaked in a vitamin solution. The soaked wheat was allowed to dry. Then the wheat was distributed as follows:

4 vials received each 4 gm of unaltered wheat

4 " " " 4 gm of pearled wheat, dry

4 " " " 4 gm of pearled wheat soaked in water and then dried

3 " " " 4 gm of pearled wheat vitaminized

1 " " " 3.2 gm " " "

The vitamin solution contained the following vitamins:

Pyridoxin, 0.0097 gm; Thiamin, 0.0073 gm;

Calcium pantothenate, 0.0105 gm;

Niacin, 0.0070 gm; Riboflavin, 0.0092 gm.

These were tipped into water and made up to approximately 10 ml. The riboflavin did not completely dissolve. This solution was poured on 16 gm of the pearled wheat in a petri dish. It was covered and left overnight to allow the solution to soak in. Then it was rapidly dried in an oven at 47°C. Later it was removed to the vials. This treated grain thus had about 0.0100 gm of each vitamin on the 16 gm of grain, or $\frac{0.0100}{16}$* gm vitamin / gm grain, or 625 / gm of grain. (Blewett and Fraenkel, 1944, used 500 /gm of food stuff.) The water-soaked grain was similarly treated but, of course, contained no vitamins.

All the vials were the same size and shape. They were supplied with clean wire gauze covers held in place by medical tape.

Later 12 weevils were placed in each vial. These weevils came from a MW culture started 3 Feb. '55, or from a GG culture started

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* i.e. 0.0070 gm. to 0.0105 gm. or, approximately, 0.0100 gm.

3, Feb. '55. All weevils were, in appearance, typical of their strain. Half the total number of vials contained MW strain and half contained GG strain. There were two vials of each treatment. Thus a total of 24 weevils of each strain was subjected to each treatment. The arrangement can be seen from Table XIII which also gives the results. The experiment was set up on 31st May, 1955. The original weevils were removed from each vial and sexed on the 14th June, 1955; and the progeny of these weevils which were emerging from the grains was counted on 14 July, 1955.

One of each of the pairs of treatments was selected at random for weighing the weevils. The weevils were removed from the vials, washed in distilled water, mopped dry, put in a clean container and then later removed at random, 5 at a time, for weighing. Each weight in the table is the mean of four lots of five weevils. To counteract personal and climatic factors and because weighing extended over two days, weevils from two of the treatments were weighed some on each of the two days.

Because one lot of GG had been reared on only 3.2 gm of vitaminised wheat, steps were taken to allow for this. Some weevils from this vial were weighed and compared with the corresponding GG on dry pearled. These adjusted figures are given in brackets in the table.

E.g. 7.2 gm (4 + 3.2) wheat yields 91 weevils
3 gm " " $91 \times \frac{8}{7.2} = 101$

The mean weights of the adjusted figures incorporated the means of both sets of weighings. Results are given in Table XIII.

The results of the five simple experiments can be summarized as follows:

First Experiment (Table IX)

Strain MW at a distinct disadvantage on pearled wheat

Strain GG able to breed normally on pearled wheat

Second Experiment (Table X)

Strain MW not able to breed normally on pearled wheat

Strain GG not effected

Third Experiment (Table XI)

Pearled wheat is improved as a diet for MW strain if "improved" by a massive dose of B vitamins

Fourth Experiment (Table XII)

Pearled wheat an unsatisfactory diet for strains MW and GG but particularly for MW

Fifth Experiment

It will be seen (Table XIII) that the numbers of weevils produced (columns 5 and 6) on the different diets do not indicate any significant trend (except for MW on vitaminised pearled wheat), but if the weights of the progeny in mgm per progeny weevil or in mgm total progeny per parent weevil are considered, then more clearly marked trends are discernible. Nonetheless, it was not possible to allow for the differences in the initial sex ratio among the parent weevils nor for the differences in numbers of females per gm of grain: and both these factors may have influenced the results. However, it does seem permissable to draw certain conclusions. There is very strong evidence that pearled wheat becomes a much improved dietary substrate for the MW strain after vitamin treatment. There is some evidence that

pearled wheat is a poorer dietary substrate than unaltered wheat for both the GG and the MW strains. There is some very slight evidence that vitaminized pearled wheat is not a good dietary source for the GG strain.

It is suggested that future research might profitably be pursued by carrying out elaborate experiments of factorial design (see, for example, Yates, 1937: and Musgrave and Mitton, 1950) on the nutritional requirements of S. oryza and several strains of S. granarius, so that the various factors of sex ratio, females/gm of grain, effect of vitamins, etc., might all be compensated for. It seems doubtful, however, that a single investigator could handle all the experimental manipulations that might simultaneously be needed.

V. DISCUSSION

Much of the study of insect microbiology has been complex and perhaps, in some respects, unrewarding because it has so often been found difficult or impossible to study the micro-organisms of the partnerships separately from the insect hosts. This has been particularly the case as regards those micro-organisms living in close association with the insects and apparently of benefit to them. This situation seems to have led one investigator to claim that the micro-organisms are artifacts or cell particles or mitochondria , (Lanham, 1952). There is, however, as has been seen in the present work, some good evidence that, at least , the curious thread-like bodies found in the mycetomes of the larvae and in certain adult organs of the weevils S. oryza and S. granarius are indeed living organisms. First, in the mycetomes they are present in great numbers. Surely, in greater numbers than could reasonably be expected if they were mitochondria? Second, the curious manner in which the micro-organisms appeared to fill the mycetocytes so that an almost cyst-like or sporangia-like appearance was produced was hardly in keeping with their being mitochondria. Third, their general behaviour as regards spore formation and the existence of apparent involution forms suggests they are living organisms. Fourth, their whole behaviour, their reactions to strong saline, their ability to remain intact, if not alive, in nutrient solutions, and even their seemingly anomalous behaviour in tetrazolium salt are all indicative of their being living organisms. Fifth, there are, moreover, clear indications that they reproduce for they seemingly

increase in numbers from the egg stage to the full grown larval stage of the host insect: and they have been observed breaking up into small spore-like bodies. Sixth, the occurrence of internal granules in the mycetomal organisms similar to those often seen in bacteria and sometimes suggestive of nuclei is an additional indication that the structures are micro-organisms. Finally, most other investigators have regarded them as micro-organisms. So that there is a long scientific tradition for so doing.

The critical attitude of Lanham (1952) who employed the electron microscope, as well as new histochemical methods for detecting desoxyribo-nucleic acid, to examine critically certain cellular inclusions that had long been regarded as micro-organisms in aphids and concluded that the supposed micro-organisms were only cellular inclusions, should be weighed against the opinions of Trager (1952), Peklo (1953), and Buchner (1953), who are authorities in the field; and the critical work of Riszki (1954), who found desoxyribonucleic acid in the morphologically similar micro-organisms in the cockroach fat-body. Then, too, the mycetomal micro-organisms studied in this present investigation are very different in appearance from the globular structures in aphids and cockroaches.

If the weevil organisms are living organisms, why can they not be easily cultured? Possibly Gier (1947) is correct when he suggests that some of the micro-organisms associated with insects exist in an association so intimate that the culture of the organisms outside a living insect will be possible only after considerable advances have been made in the techniques of insect tissue culture. It is worth noting that certain parasites such as the malaria parasite of man, which is

admittedly a Protozoan, have not yet been successfully cultured outside the human blood stream or the mosquito vector; others, like many of the viruses, need host tissue *in vitro*.

It is, then, of interest to speculate how the weevils and the micro-organisms became so intimately associated and how the mycetome became inhabited. This question is rendered more intriguing by the very restricted method of transfer of the micro-organisms. That infection does not occur via the mouth; that the micro-organisms are intimately associated with a special tissue; and that the micro-organisms have proved so far to be impossible to culture successfully *in vitro* are strong evidences for thinking that the association is, from the evolutionary viewpoint, a very old one. Nonetheless, it seems likely that the original infection was via the mouth, probably from some food material. Perhaps this infection gave competitive advantages in the conquest of a new ecological niche. Then, with the recent custom of universal grain storage and shipment by man, the weevils would have become adapted (as they undoubtedly are) to the specialised environments involved. The occurrence of different strains of weevils may perhaps be explained by differing climatic factors or unusually high environmental temperatures, (e.g., overheating in stored grain, intense heat in ships' holds, etc.), causing the eradication of the micro-organisms with the consequent appearance of a different strain of weevil. However, perhaps certain strains of weevils never were infected with mycetomal micro-organisms. Again some strains of weevils may have evolved biochemically to a point where the products of metabolism completely or partially inhibit the development of the micro-organisms.

But if it were a matter of inhibition one would expect more evidence of a genetical ratio as a result of crosses between the strains rather than the evidence of simple congenital infection which has been demonstrated experimentally. One is therefore led to theorise that at some period in their evolutionary history the weevils became orally infected by a micro-organism from its food. Perhaps the micro-organism was in a particularly suitable phase at the time of ingestion or perhaps the organism was relatively free-living saprophyte or facultative parasite has become extinct and exists now only as an obligate associate in the weevils. The Sitophilus weevils are not the only insects with which micro-organisms are associated and it is likely that some similar theory of initial infection must be developed for each species of insect. One point of special interest in this association of micro-organisms with insects is the development of the peculiar structure - the mycetome.

In the two species of weevils under consideration in this work, the mycetome is a larval structure. How did the mycetome originate? Does it represent a response by the insect to the invasion of a foreign organism - a kind of hypertrophied growth stimulated in the insect? Alternatively does it represent a mass of spore-like bodies developing endogenously with the globular mother cells represented by the mycetocytes? The tracheal supply to the mycetomes is extensive and indicates oxygen want in that region, for Wigglesworth (1954) has shown recently that tracheae will migrate, even during the life-time of the individual insect, at least small distances, to regions of oxygen want. Whatever the mycetome in fact represents morphologically, it is remarkable that it should break up during metamorphosis and that the individual mycetocytes

should migrate posteriorly and lodge in the developing midgut. As metamorphosis is known to be initiated by hormones secreted from the head of the insect, one may reasonably suppose that the same hormones may regulate the movements of the mycetocytes. However, it proved to be impossible to show that the head hormones had any such effect. It did seem in the present work, however, that the micro-organisms underwent some kind of reproductive phase during the pupal stage as it was then that many of the small spore-like bodies were seen. The tracheal supply to the adult midgut coeca is good which again suggests that the micro - organisms are aerobic. Where they obtain oxygen during passage from the mycetome to the midgut, however, is a matter for speculation. However, many micro-organisms are facultative anaerobes.

An alternative and more fascinating theory is that the mycetome, rather than breaking up under the influence of hormones causing metamorphosis, is itself a gland secreting a hormone. Some reference to some recent findings about the factors controlling metamorphosis in insects is of considerable significance here; for (vide Bodenstein in Roeder's text) in the few insects that have been extensively investigated certain neuro-secretory cells near the brain produce hormones which under certain precisely regulated conditions affect a gland or glands in the thorax of the insect. The thoracic gland secretion appears to be a hormone that plays an absolutely essential part in the metamorphosis of the insect. In the larvae of certain Lepidoptera there is a prothoracic gland of this kind. It is described as comprised of large cells richly supplied with tracheae. In Sialis, a Neuropteran, a gland of similar function has been described in the third thoracic and first abdominal

segments. In the bug Rhodnius, (Wigglesworth, quoted in Roeder, p.909) has recently found some glandular material in the prothoracic region which he considers to be of similar function to the prothoracic glands of the Lepidopters.

To date there seems to be no work of this kind on the Coleoptera. But it is a very reasonable assumption that some similar glands fulfill a similar function. Thus it is a reasonable premise to admit the existence of glands of this kind in the Sitophilus weevils. The mycetome in the larva is in the thoracic region, its cells are large and are well supplied with tracheae and the mycetome is obviously "concerned" with metamorphosis. It is therefore suggested that future research may show that the mycetomes of many Coleoptera are, in fact, endocrine glands of essential function that have become adopted as a location for some micro-organisms, perhaps originally parasitic, that have, in the course of evolution, become intimately associated with the host weevil. The micro-organisms may have a stimulating effect on the growth of the glands for they seem to be larger when inhabited. If this idea is correct then the whole matter of cultivation of all such micro-organisms "in vitro" will very likely depend on precise and delicate adjustments in an artificial medium containing specific hormones from insect material.

Some evidence has already been submitted (p. 37) that suggests that the micro-organisms might be Actinomycetes. Actinomycetes are common in the soil but they are also known to occur in other habitats: the natural habitat for Nocardia is grass (Waksman, 1950, quoting Pipper and Pullinger). Is it possible, then, that the Sitophilus weevils originally were infected from some member of the Graminese on which they were feeding?

Some of the micro-organisms found in insects have been referred to as diphtheroid bacteria. Wigglesworth (1936) at first referred to the micro-organisms of Rhodnius in this way and only later, after reading Erikson, did he suspect and then show them to be an Actinomycete (Brecher and Wigglesworth, 1944). Perhaps future research will show that some of the micro-organisms in other insects are members of the Actinomycetes.

Is it likely to be the same species of micro organism in the two species of weevils? Clearly there can be no accurate answer to this until it is possible to cultivate the micro-organisms on artificial media. The size differences found in the present investigation are not, of course, guarantees of specific differences. But the association between the weevils and the micro-organisms is undoubtedly very intimate, which suggests that it is an association that likely arose early in the evolutionary development of the two species of weevils. Thus, if the two species of weevils have diverged, likely, their associated micro-organisms have diverged too. Such a close adaptation might well prevent them from adopting any kind of life in a different environment.

The micro-organisms may be merely useless and harmless associates, parasites that the weevils effectively keep under control or true mutualists conferring benefit upon the insects in exchange for shelter. While these are obviously anthropomorphic expressions, it is generally conceded that they do serve to describe rather subtle distinctions in the relationships that may exist between two associates. It has been commonly accepted that the insect derives some benefit from the organisms, though among the dissidents from this view was Mansour. If benefits are conferred, those most likely would be the production of antibiotics by

the micro-organisms or the production of some special enzyme, co-enzyme or vitamin. The most likely vitamin requirement would be the B complex, for it has now been established (vide Fulton, 1949) that animals living on a high carbohydrate diet require vitamin B complex for the proper digestion and assimilation of this material. Fraenkel (1952) and his associates have shown that the micro-organisms do, indeed, supply vitamins of the B complex to the host insect in certain other species.

Experimental evidence produced in the present work indicates that the adults of neither species of weevils are individually seriously handicapped by the absence of micro-organisms, yet there are very strong indications (p. 42) that the treatment of rice weevils in a manner that apparently removes their micro-organisms seriously interferes either with their reproductive potential or else with the development of their larvae. S. granarius is not thus affected to the same degree. Indeed, as there seem to be micro-organisms free strains, this is not surprising. Thus we may theorise that while S. granarius can, under conditions of normal diet, function without micro-organisms, S. oryza apparently cannot. The major difference between the two species is the possession of membranous wings under the elytra in S. oryza. Wing development is thought to be a function of nutrition (Wigglesworth, 1950). Can it be that the greater dependence of S. oryza on micro-organisms is due to its development of wings?

One anomaly in the results calls for some comment. If the antibiotic Terramycin causes removal of micro-organisms from the living insect, how is it that the presence of Terramycin and Penicillin in the hanging drop cultures seems to have either no effect on the micro-organisms

or else a preserving effect? The immediately forthcoming explanation for this apparent anomaly is that the antibiotics function differently in different environments. The antibiotics are ineffective or perhaps insufficient in amount to inhibit the organisms "in vitro" in the synthetic media tried whereas "in vivo" they are able effectively to disturb the general metabolic relationship between the weevils and their micro-organisms. Then, too, it is known that Penicillin in certain concentrations may have a bacteriastatic effect by preventing micro-organisms increasing in numbers by fission. Thus in vivo in the weevils where there must needs be a great multiplication from egg to adult an antibiotic might have a very marked effect, while in vitro, the reduction or inhibition of fission would not be associated with any effect on the weevil, but concurrently might serve to defend the mycetomal micro-organisms from the lytic effects of other micro-organisms that might be present.

There certainly seems to be a well maintained balance between the insect and its micro-organisms. For while there was considerable variation in the numbers of micro-organisms harboured by the weevils there was never any evidence of ill health in the weevils as a result of infection - in S. granarius, the situation seemed to be very much the contrary, the larger, darker, more massive weevils being those from the strain with micro-organisms. Nor was any impression obtained that the micro-organisms were harming the insect tissues.

In S. granarius a second type of organism was frequently found. It was very much smaller than the standard thread-like form and the relationships between the two forms were only superficially investigated

for reason of limitations of optical resolution and cultural techniques.

Again in some specimens of S. granarius of the micro-organism-free strain, in mycetocyte-like structures in the ovarioles there could be seen curious amoeboid masses in the places where one would have expected to see the thread-like micro-organisms.

The discovery of the two strains from London, Ontario, that seemed to be free of micro-organisms is of great interest. Mansour (1935b) had claimed that there was an Egyptian variety that was free of micro-organisms; and Koch, who seemed to accept the existence of such a strain, mentioned that Zacher had described a strain from Egypt that was distinguishable as smaller in size and lighter in colour than the usual strain. He called this strain variety africana. It seems possible that the MW strain that has been used as experimental material in this investigation may be the same as, or similar to, the variety africana of Zacher. It thus seems that Mansour's observation was correct.

It is evident that at least two distinct strains of S. granarius weevils, MW and GG, separable by certain observable characteristics, have been studied in the present work. But while there is striking evidence against the Null Hypothesis, "that S. granarius does not harbour mycetomal micro-organisms", the evidence against the Null Hypothesis, "that the MW strain of S. granarius harbours mycetomal micro-organisms", is not so strong. The fact that most of the individuals of the MW strain examined had few or no micro-organisms* might indicate one or more of several circumstances: (i) the full development of the micro-organisms might be inhibited; (ii) micro-organisms might be present in an undetectable form; (iii) micro-organisms might be so few in numbers

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* In this paragraph the word 'micro-organism' is used in the very restricted sense of one typical of the GG strain.

as to be virtually undetectable or, (iv) the micro-organisms might be absent. But no matter what the exact situation may be there is very good evidence for the contention that the flora of thread-like mycetomal micro-organisms in the strains MW and GG is quantitatively and perhaps qualitatively different. Moreover, the evidence in favour of the MW strain as one harbouring micro-organisms is very poor indeed. The experiment (p. 51) in crossing the strains also suggests that there is no inhibition of the full development of the micro-organisms. Simple congenital infection via the female was indicated. The existence of the MW strain certainly affords an explanation of Mansour's observations, (1935a, 1935b.).

In the very simple nutritional experiments that were conducted the results indicated that pearled wheat was less satisfactory than plain wheat for strains MW and GG of Sitophilus granarius. It was also clear that pearled wheat treated with vitamins was greatly improved as a dietary substrate for the MW strain. But there was no clear evidence of this with the GG strain. Caution must be exercised in discussing the results of such simple experiments. However, it is possible that the micro-organisms in the weevils of the GG strain do not enable their hosts to live optimally on grain robbed of some of its B vitamins. Perhaps, too, the vitamins supplied were not those required in spite of the fact that they seemed to satisfy much of the needs of the MW strain, for the GG strain might have different requirements. Perhaps, because of its slighter build and lighter colour, the MW strain needs a less complicated diet than the GG strain. If the MW strain is free of mycetomal micro-organisms, clearly it is not dependent upon them; it seems, too, that it

is adaptable to conditions of deficient diet to much the same degree as the GG strain. Indeed, one may suppose that in order to survive as a strain in nature it would be necessary to be adaptable to deficient diets. The precise part played by the mycetomal micro-organisms in the economy of the weevils thus remains doubtful, but clearly they are associated with larger, darker weevils.

It is, however, unlikely that the existence of the MW and LG strains offers an explanation of the discrepancy between the results of feeding Terramycin to the two species of weevil as described here (p. 39), and published as a short paper by Musgrave and Miller (1951), and the results of Steinhaus and Bell (1953), who claimed that the two species of weevils were not differently affected by the Terramycin. The results of Steinhaus and Bell must, however, be discounted, for they fed the weevils a dose of Terramycin which (in their hands) was sufficient to cause 100% response (in this case, mortality) in both species of weevils. It is well known that while two species of organisms A & B may be killed by a dose x of a substance, this dose may be that just sufficient to kill A and, simultaneously, much more than sufficient to kill B. We could fantastically say that the dose x caused 100% mortality in A and 150% mortality in B. This is one of the reasons why the $L.D_{50}$ (or median lethal dose or the dose to give 50% mortality) is respected in modern bioassay, particularly as a basis of comparison. No reliable comparisons can be made at the 100% response level. The matter is briefly discussed by Finney (1947).

However, the results of the Terramycin experiment just mentioned attracted attention from other quarters outside Canada. Indeed, Buchner

(1953) discussed them in the latest edition of his masterly work on symbiosis. He thought that it should be determined whether the Terramycin did remove the micro-organisms and contended further that it would not be possible to determine with certainty if the mycetomal micro-organisms benefit the weevils until weevils freed of micro organisms could be obtained, and compared both with those harbouring micro-organisms and with the Egyptian variety.

The present work has, it is claimed, clarified considerably the whole complicated association of Sitophilus oryza and Sitophilus granarius and their mycetomal micro-organisms. Apart from correcting the misleading statements in standard works by Jeannel (1949), Steinhaus (1946) and Wigglesworth (1950) about the absence of mycetomal micro-organisms from all Sitophilus granarius, it has suggested an explanation of Mansour's finding of the Egyptian strain and produced evidence that a similar strain exists in Canada. Moreover evidence has been produced to confirm that the mycetomal micro-organisms are indeed living entities; means of keeping them alive outside the insect have been devised; and their mode of transfer congenitally through the female has been demonstrated. Then, too, it has in no small way contributed towards fulfilling the requirements of Buchner (1953) just mentioned, for it has shown that differences in the population of mycetomal micro-organisms in Sitophilus granarius are associated with differences in the general facies of the host insects such that at least two strains thereof can be separated; moreover, evidence has been obtained that Terramycin treatment of the grain did cause loss of mycetomal micro-organisms in weevils that fed on it, and that Sitophilus oryza was apparently more adversely affected by this than S. granarius.

VI. CONCLUSIONS

Certain thread-like structures occurring in the weevils Sitophilus oryza and Sitophilus granarius are micro-organisms. They are of doubtful affinity but there is clear evidence of some kind of pleomorphic life cycle, and indications that they may be Actinomycetes, possibly of the genus Nocardia. Their exact physiological role in the weevils' economy is obscure, but there is evidence that they are of some nutritional benefit, particularly to Sitophilus oryza. The organisms are passed congenitally from one generation of weevils to another only through the female. There is no evidence of oral infection. S. granarius is able to live without the micro-organisms for it is not seriously affected by their removal; and, moreover, strains of this species exist in which most of the individuals have no mycetomal micro-organisms or very few. These strains are distinguishable to a considerable degree by their size, shape and colour. It is possible that in them the full development of the micro-organisms is inhibited, but evidence is in favour of a simple lack of infection. Because of the intimate nature of the association between the weevils and their micro-organisms it is unlikely that the latter will be successfully cultured until the technique of insect tissue culture is more advanced. However, the micro-organisms may be maintained alive for long periods outside the insect, in hanging drop cultures containing synthetic nutrient media. It is probable that the association between Sitophilus oryza and its micro-organisms is truly mutualistic.

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TABLE I

THE EFFECT OF TERRAMYCIN TREATED

GRAIN ON THE TWO SPECIES OF WEEVILS

	SITOPHILUS ORYZA								SITOPHILUS GRANARIUS							
	Fed terramycin-treated wheat				Fed untreated wheat (water)				Fed terramycin-treated wheat				Fed untreated wheat (water)			
No. originally	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
No.At End: Alive:	3	6	4	4	30	94	85	70	27	37	19	28	48	57	50	51
Dead:	23	19	17	20	23	4	0	9	12	11	17	13	5	6	6	6
TOTAL:	26	25	21	24	53	98	85	79	39	48	36	41	53	63	56	57
Increase in Population	6	5	1	4	33	78	65	59	19	28	16	21	33	43	36	37
Condition of Grain	C	C	C	Mean	B	B	B	Mean	B	B	B	Mean	A	A	A	Mean

Condition of grain:

A - very well eaten: only husks and powder.
B - well eaten.
C - eaten slightly.

TABLE II

THE EFFECT OF DIFFERENT DOSES OF TERRAMYCIN
ON THE TWO SPECIES OF WEEVILS

	SITOPHILUS ORYZA												SITOPHILUS GRANARIUS											
	Fed Terramycin Wheat (low dose)				Fed Terramycin Wheat (high dose)				Fed Untreated Wheat				Fed Terramycin Wheat (low dose)				Fed Terramycin Wheat (high dose)				Fed Untreated Wheat			
No. of Humidor	4	6	5		4	6	5		4	6	5		1	2	3		1	2	3		1	2	3	
Number of Weevils at start	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	
Number of weevils at end Alive	55	59	46		25	54	22		61	67	50		41	41	39		38	36	38		44	39	45	
Dead	5	1	7		6	2	7		8	3	12		1	7	5		6	2	9		3	6	3	
Total	60	60	53		31	56	29		69	70	62		42	48	44		44	38	47		47	45	48	
Population Increase	40	40	33	38	11	36	9	19	49	50	42	47	22	28	24	25	24	18	27	23	27	25	28	27
Condition of Grain																								
Replicate	1	2	3	M	1	2	3	M	1	2	3	M	1	2	3	M	1	2	3	M	1	2	3	M

High Dose 1000 grs/ton
Low Dose 10 grs/ton

TABLE III

THE EFFECT OF TERRAMYCIN TREATED GRAIN ON THE PRESENCE OF MYCETOMAL MICRO-ORGANISMS INSIDE THE WEEVILS

S. oryza fed Terramycin-treated grain					*S. oryza* fed ordinary grain					*S. granarius* fed Terramycin treated grain					*S. granarius* fed ordinary grain				
No	Sex	Date	Gut	Gon	No	Sex	Date	Gut	Gon	No	Sex	Date	Gut	Gon	No.	Sex	Date	Gut	Gon
1	♀	19vi52	?	P	1	♂	19vi52	P	-	1	♀	20vi52	?	P	1	♀	23vi52	?	P
2	♀	19vi52	P	-	2	♀	19vi52	P	P	2	♀	23vi52	?	P	2	♂	23vi52	P?	-
3	♀	20vi52	P	P	3	♂	19vi52	P	-	3	♂	15vii52	N	N	3	♂	23vi52	N	-
4	♂	23vi52	P	-	4	♂	25vi52	P	-	4	♂	11ix52	N	N	4	♂	23vi52	N	-
5	♂	27vi52	P	-	5	?	26vi52	P		5	♂	20x52	N	?	5	♀	15vii52	P	P
6	♀	2vii52	P	P	6	♀	14vii52	P	P	6	♀	20x52	N	?	6	♂	16vii52	?	?
7	♂	21vii52	P?	-	7	♂	14vii52	P	N	7	♂	20x52	N	-	7	♀	11ix52	P	P
8	♀	23vii52	P?	-	8	♂	14vii52	P	N	8	♂	20x52	?	N	8	♀	20x52	P	P
9	♀	11vii152	N	N	9	♂	15vii52	P	N	9									
10	♀	15vii152	N	N	10	♂	26vii152	P	N										
11	♂	26vii152	N	-	11	♀	26vii152	P	P										
12	♂	26vii152	N	-	12	?	27vii152	P	?										
13	♀	27vii152	N	N	13	♂	28viii52	P	-										
14	?	27vii152	N	N	14	♀	28vii152	P	P										
15	♂	27vi152	N	N	15	♀	29viii52	P	P										
16	♀	29vii152	N	N															

TABLE IV

AS TABLE III. THE RESULTS OF A SECOND SERIES OF TESTS

S. ORYZA									S. GRANARIUS								
Fed Terramycin treated Grain					Fed Ordinary Grain				Fed Terramycin treated Grain					Fed Ordinary Grain			
No.	Date	Sex	Gut	Gonad	No.	Date	Sex	Gut	Gonad	No.	Date	Sex	Gut	Gonad	No.	Date	Sex	Gut	Gonad
1	21vi1152	♂	P	-	1	21vi1152	♀	P	P	1	6ix	♀	N	N	1	6ix	♂	P	N
2	21x52	♀	N	N	2	21x52	♂	P	-	2	8ix	♀	?	P	2	8ix	♂	P	-
3	21x52	♀	P	P	3	21x52	♀	P	P	3	10ix	♀	N	?	3	10ix	♂	?	N
4	6ix52	♀	N	N	4	5ix52	♀	?	?	4	10ix	♀	N	P	4	10ix	♀	N	P
5	6ix52	♂	N	-	5	6ix52	♀	P	P	5	11ix	♀	?	?	5	11ix	♂	P	-
6	3 x52	♀	N	N	6	6ix52	♂	P	-	6	8x	♀	?	?	6	24ix	♀	?	P
7	22x52	♀	N	N	7	241x52	♀	P	P	7	8x	♀	P	P	7	24ix	♂	P	-
8	22x52	♀	N	N	8	241x52	♀	P	P	8	9x	♀	N	N					
9	24x52	♀	N	N	9	241x52	♀	P	P	9	9x	♂	N	N					
10	24x52	♂	N	N	10	3x52	♀	P	N										
11	29x52	♂	N	-	11	3x52	♀	P	-										
12	29x52	♂	N	-	12	22x52	♀	P	P										
13	22x52	♂	N	N															
14	22x52	♀	N	N															

P - Positive

N - Negative

TABLE V

THE POPULATION INCREASES OCCURRING IN THE SAMPLE POPULATIONS UNDER TEST IN THE EXPERIMENTS GIVEN IN TABLE III

	ORIGINAL	TOTAL	INCREASE
Granarius untreated	25	34	9
Granarius treated	25	27	2
Oryza untreated	25	53	28
Oryza treated	25	30	5
Granarius untreated	20	32	12
Granarius treated	20	26	6
Oryza untreated	20	46	26
Oryza treated	20	25	5

TABLE VI

PRESENCE OF MYCETOMAL MICRO-ORGANISMS

IN WEEVILS OF DIFFERENT AGES

DATE OF REMOVAL FROM PARENT CULTURE	SPECIMENS EXAMINED DURING MONTHS SHOWN WITH RESULTS				
	APRIL	MAY	JUNE	JULY	AUGUST
13 MARCH	4 +	2 + 2 dead	4 + 1 -	2 -	2 + 1 dead
16 MARCH		2 + 2 -		1 + 1 - 1 dead	1 + 1 dead
20 MARCH		4 +	1 +	2 +	4 + 1 - 1 dead
24 MARCH		1 + 1 -	3 + 2 dead	2 + 7 dead	4 + 2 - 3 dead
9 APRIL		6 + 3 - 3 dead	4 + 1 - 2 dead	2 +	2 + 1 dead

TABLE VII

WEIGHTS OF WEEVILS OF DIFFERENT STRAINS

SITOPHILUS GRANARIUS — 10 specimens weighed at a time

Figures given below are mean weights of ten weevils in mgm/weevil

Strain GG	Strain MW
2.57	2.00
2.48	2.27
2.80	2.16
3.00	2.08
2.70	2.05
2.71 Means	2.112

Sums of squares of deviations:

for MW = 0.0395
for GG = 0.1648

$\bar{x}_{GG} = 2.71$ $\qquad$ $\bar{x}_{MW} = 2.112$

$$S_{GG} = \sqrt{\frac{0.1648}{4}} \qquad S_{MW} = \sqrt{\frac{0.0395}{4}}$$

$$\bar{x}_{GG} - \bar{x}_{MW} = 2.71 - 2.112 = 0.598$$

$$S_D = \sqrt{\frac{\left(\sqrt{\frac{0.1648}{4}}\right)^2 + \left(\sqrt{\frac{0.0395}{4}}\right)^2}{5}}$$

$$= 0.1109$$

$$t = \frac{0.598}{0.1109} = 5.393$$

From tables t at 0.05 level of probability with 8 degrees of freedom = 2.306

Necessary difference between means for significance = 2.306 x 0.1109 = 0.2617

The mean weights of the two strains of weevils are therefore significantly different at the 0.05 level.

TABLE VIII

WEIGHTS OF WEEVILS OF DIFFERENT STRAINS

SITOPHILUS GRANARIUS — 3 specimens weighed at a time

Figures given below are mean weights of three weevils in mgm/weevil

	Strain MW	Strain LG	Strain GG
	2.03	2.60	2.53
	2.23	2.66	3.16
	2.00	2.80	2.73
	1.93	2.73	3.33
	2.03	2.96	3.26
	2.23	2.90	3.066
		2.93	2.63
		2.73	
Mean:	2.075	2.79	2.958

The weevils in all the weighing experiments were removed from cultures in comparable condition for each set of weighings and there was no reason for thinking that any unconscious bias had entered into the taking of the observations.

TABLE IX

FEEDING STRAINS MW and GG PEARLED AND ORDINARY WHEAT

Experiment set up: 30-31 July 1954. Final count: 15 Oct 1954.

		Number originally	Number finally	Population increase	% increase
Plain Wheat	MW strain	7	56	49	700
	GG strain	7	59	52	743
Pearled Wheat	MW strain	7	25	18	72
	GG strain	7	64	57	814

TABLE X

FEEDING STRAINS GG and MW PEARLED AND ORDINARY WHEAT

		Originally (30 Sep 54)	Removed 7 Oct 54	Finally (17 Nov 54)	Increase	% Increase	Weight mgm/ weevil
Plain Wheat	MW	25	11♂ 14♀	50	50	200	2.04
	GG	25	14♂ 9♀	26	26	113	2.70
Pearled Wheat	MW	25	12♂ 13♀	29	29	116	1.50
	GG	25	12♂ 12♀	42	42	175	2.07

Less than half of the GG weevils on plain wheat were females. This might have affected the increase as it is reported by Richards (1947) that an excessive number of males in a culture has a depressing effect on the extent of oviposition.

TABLE XI

EFFECT OF B VITAMINS ON IMPROVING PEARLED WHEAT

AS A DIET FOR MW STRAIN

Vitamins: in 5 ml distilled water

Niacin 3.6 mgm;
Thiamine 2.0 mgm;
Riboflavin 2.3 mgm;
Pyridoxin 5.1 mgm;
Calcium pentothenate 3.8 mgm.

	Originally (25 Oct 54)	Removed 1 Nov 54	Finally (3 Dec 54)	Increase	% Increase	Weight mgm/ Weevil
MW strain with vits	20 20	11♀7♂2?	31	61	305	1.50
water soaked	20	12♀ 7♂1?	29	89	145	1.60
GG strain water soaked	20	11♀ 8♂	23	23	121	2.30
dry	20	13♀ 6♂1?	53	53	265	1.80

ALL WHEAT PEARLED

TABLE XII

PEARLED WHEAT AS A DIETARY SOURCE FOR STRAINS MW and GG

Strain	Treatment of grain	Original number of weevils		Final increase	Increase/ female
MW	unaltered	1♂	5♀	30	6.0
MW	pearled	1♂	5♀	3	0.6
GG	unaltered	4♂	6♀	49	8.2
GG	pearled	4♂	5♀	12	2.4

4 gm of grain per vial

TABLE XIII

STRAINS MW and GG of S. GRANARIUS FED PEARLED AND VITAMINIZED PEARLED WHEAT

STRAIN (1)	GRAIN TREATMENT (2)	ORIGINAL POPULATION (3)	REMOVED 14 Jun 55 (4)	FINAL POPULATION (5)	PROGENY PER FEMALE (6)	MEAN WT PROGENY (7)	WT. DIFF. % (8)	WT TOTAL PROGENY/FEM (9)	DIFF. (10)
GG	Unaltered	24	10♀13♂1?	82	8.2	2.73	-	22.39	
MW	Unaltered	24	11♀13♂1?	124	11.27	2.153	-	17.24	
GG	Pearled dry	24	13♀12♂	106	8.4	2.26	-17.27	18.99	-15.19%
MW	Pearled dry	24	18♀ 6♂	139	7.8	1.78	-17.33	13.89	-19.43%
GG	Pearled soaked	24	15♀11♂	123	8.3	-	-		
MW	Pearled soaked	24	18♀6♂	93	5.3	-	-		
GG	Pearled vit	24	15♀10♂	91 (101)	6.1 (6.74)	2.09 (2.18)	No Increase	12.26 (14.70)	No Increase
MW	Pearled vit	24	20♀ 3♂1?	179	8.9	1.83	2.809	16.29	17.28%

NOTES: (a) Column 7: weights are mgm/weevil.

(b) Column 8: shows decrease in weight, as a percentage, between those fed pearled and plain wheat, and increase of those fed vitaminized pearled over those fed pearled.

(c) Column 10: shows similar differences in weight of total progeny.

TABLE XIV

COMPOSITION OF EARLE'S SOLUTION

(From Parker; Methods of Tissue Culture, 2nd Edition)

Sodium chloride	6.80 gm.
Potassium chloride	0.04 gm.
Calcium chloride	0.20 gm.
Magnesium sulfate	0.20 gm.
Sodium phosphate monobasic	0.14 gm.
Sodium bicarbonate	2.20 gm.
Glucose	1.00 gm.
Water (glass distilled)	to make 1 liter.

Composition of Morgan, Morton, and Parker's Medium No. 199

(From Parker: Methods of Tissue Culture 2nd Edition)

All constituents in Mg. per 1000 ml.

l - Arginine	70.0	Thiamin	0.010
l - Histidine	20.0	Riboflavin	0.010
l - Lysine	70.0	Pyridoxin	0.025
l - Tyrosine	40.0	Pyridoxal	0.025
dl - Tryptophane	20.0	Niacin	0.025
dl - Phenylaline	50.0	Niacinamide	0.025
l - Cystine	20.0	Pantothenate	0.01
dl - Methionine	30.0	Biotin	0.01
dl - Serine	30.0	Folic acid	0.01
dl - Threonine	60.0	Choline	0.50
dl - Leucine	120.0	Inositol	0.05
dl - Isoleucine	40.0	p-Aminobenzoic acid	0.05
dl - Valine	50.0	Vitamin A	0.10
dl - Glutamic acid	150.0	Calciferol	0.10
dl - Aspartic acid	60.0	Menadione	0.01
dl - Alanine	50.0	-Tocopherol phosphate	0.01
l - Proline	40.0	Ascorbic acid	0.05
l - Hydroxyproline	10.0	Glutathione	0.05
Glycine	50.0	Cholesterol	0.2
Cysteine	0.1	Tween 80	20.0
Adenine	10.0	Sodium acetate	50.0
Guanine	0.3	l-Glutamine	100.0
Xanthine	0.3	Adenosine triphosphate	10.0
Hypoxanthine	0.3	Adenylic acid	0.2
Thymine	0.3	Ferric nitrate	0.1
Uracil	0.3	Ribose	0.5
		Desoxyribose	0.5

APPENDIX I

CHRONOLOGICAL LIST OF HANGING-DROP CULTURES

Starting Date	Ref.	Observations	Termination Date
21/1/53	O/4	Oryza mycetome. Nothing of interest.	24/1/53
23/1/53	O/4	Mycetome from a very small granarius larva.	27/1/53
26/1/53	O/5	Mycetome from oryza; agar layer in the drop. Standard organisms seem to be replaced by "cocci"	?
27/1/53	O/5	Mycetome from a third stage granarius larva in a drop of dist. water, blood and fat body from a full grown larva "cocci" developed.	
9/2/53	O/6	Granarius larval mycetome in pupal body fluid. Involution forms and many tiny bodies developed.	
16/2/53	O/7	Granarius ovarioles in drop of water. No organisms!	
16/2/53	O/6	Oryza ovarioles	
9/3/53	O/9	Gut from a granarius pupa in drop of pupal blood and fat-body. 10/3/53: many "cocci"	10/3/53
10/3/53	O/10	Gut from a granarius pupa in a drop of pupal blood and fat body. Organisms were seen apparently developing from globules. Many tiny rods.	10/3/53

11/3/53	G/11	Gut and mycetome of prepupa of granarius combined in drop with gut and blood of young pupa. Many of the tiny organisms developed and gave the appearance of attacking the standard organisms; by 16/3 there were very few standard organisms and the tiny organisms seemed mostly "dead". Mould mycelium was developing.	
	G/12	Data too scanty for inclusion.	
18/3/53	G/13	Gut from granarius browning pupa into a drop of Locke's solution. Notes scanty.	
18/3/53	G/14	Gut from a white granarius pupa into Locke's solution. This gut was in an early stage of metamorphosis, before the coeca had developed. There were many standard organisms initially. 20/3: many of the tiny organisms were visible giving the impression of attacking the standard organisms. Standard organisms with jet black dots. 27/3: Tiny organisms clearly reproducing. Standard organisms apparently dying off.	2/4/53
24/3/53	G/12	Oryza: gut of white pupa in Locke's solution. Obvious contaminants developed.	25/3/53
25/3/53	G/13	Gut from a white oryza pupa. No tiny organisms sich as granarius had.	27/3/53
	G/15	Notes too fragmentary.	
25/4/53	G/16	Part of mycetome and head from a granarius prepupa. Blood and gut from a pupa in sterilised tap water. No growth or reproduction. Eventually a fungus mycelium appeared.	5/5/53
	G/17	Yielded nothing of interest.	

5/5/53	G/18	Granarius pupal brain and prepupal mycetome in a drop of Parker's No. 199. 6/5: many organisms had become globular.	8/5/53
11/5/53	O/15	Oryza in No. 199. Contaminated from the synthetic medium.	29/5/53
21/5/53	G/19	Granarius in No. 199.	29/5/53
3/6/53	G/20	Granarius gut in Earle's medium. Deteriorated rapidly.	5/6/53
26/6/53	O/16 O/16a	Two oryza cultures that produced no significant results.	
25/6/53	G/21	Granarius material mixed with a penicillin solution. On 30/6/53 some contaminants were visible.	
2/7/53	O/17	A mycetome and some pieces of nerve from an oryza prepupe were put in a drop of Terramycin solution. On 3/7/53 ordinary standard organisms apparently dividing by fission and others staying linked in chains. 7/7/53 a mass of small globular organisms. An attempt to transfer to a new slide failed.	7/7/53
16/7/53	O/19	Oryza a dual drop. One part gut, nerve and mycetome pieces of prepupal material containing abundant micro-organisms; other part, young female gonads. Both parts in Earle's. After mixing of drops there was no migration of organisms towards the gonad material. This gonad material remained in a life like condition for a considerable time.	
	O/20	Ruined in an early accident.	
24/7/53	O/21	A dual drop: one part, nerve material from a prepupa; other part, mycetome from a small larva.	
19/8/53	G/22	A dual drop. But drops were too big for proper viewing.	9/9/53

22/9/53	G/23	Mycetome from small granarius larva with some pieces of gut in Earle's Solution. Mycetome did not break. On 23/9 transferred to an ordinary slide and mycetome crushed. 28/9 many coiled types present. 15/1/54 some usual granarius organisms still present.	15/1/54
16/10/53	GO/1	A double hanging drop: part from each species. Nothing notable observed.	13/11/53
19/10/53	GO/2	A double drop in Earle's. Two mycetomes; one from each species. Many organisms in each drop. 21/10/53 Difference between two sides is now quite marked. Drops mixed. 22/10 Transferred. 14/11 both kinds of organisms can still be seen, as well as some very small organisms. 15/1/54 both kinds still distinguishable.	
23/10/53	GO/3	Double drop in Earle's on an ordinary slide: one section nervous tissue from a granarius pupa; one section, mycetome from an oryza larva. No definite evidence of influence of nerve tissue on organisms.	21/12/53
23/10/53	GO/4	An attempt to make a double drop of granarius gut and oryza brain Never a success.	13/11/53
3/11/53	GO/5	A double drop: brain etc. of a white pupa of oryza and the mycetome of a granarius larva in Earle's solution. Though at one time the nerve tissue seemed to be growing, no definite evidence was found. On 14/12/53 there were thousands of typical granarius organisms.	21/12/53
20/11/53	GO/6	Very much as GO/5. But there were only a few organisms at termination.	21/12/53

24/11/53	O/22 O/23 O/24	Preliminary experiments with drops containing antibiotics; records fragmentary.
27/11/53	O/25	Oryza mycetome from a nearly full-grown larva in Earle's solution with slice of penicillin sensitivity disc. On 3/5 it seemed that division might be occurring. On 27/1/55 the organisms in this drop seemed still alive.
27/11/53	O/26	Oryza mycetome from a nearly full-grown larva in Earle's solution with Terramycin sensitivity disc. 9/2/54 thousands of organisms. 27/1/54 apparently still alive: with mobile organisms and well developed mycetocytes filled with organisms.
8/12/53	G/25	Segment of granarius in Earle's with piece of Penicillin sensitivity disc. Many organisms took up colour from disc. 9/2/54 many typical organisms were still present.
8/12/53	G/27	Granarius material in Earle's Solution. Anterior nerve tissue from a prepupa; gut tissue from a pale brown adult (i.e. one that had not yet left the grain) 29/12/53 Many globular bodies 27/1/55 Still alive.
1/12/53	GO/7	Gut from a white granarius pupa and gut and nerve from a pale brown oryza adult. 21/12/53 Two gut pieces can be seen: one is surrounded by typical granarius organisms. The other is with coeca and is surrounded by typical oryza organisms. No special activity associated with the very healthy looking piece of nerve tissue. 2/1/54 around coeca-less gut piece are many standard granarius organisms; the field becomes more mixed further from this gut piece and then around the piece of gut with coeca are typical oryza organisms. Organisms of this slide still apparently alive on 11/3/54.

23/12/53	0/27	Oryza larval mycetome in Earle's solution with small piece from penicillin disc. (Estimated at 0.3540 units in .07 ml.) 9/2/54 Thousands of organisms, several mycetocytes.	11/3/54
23/12/53	0/28	Oryza larval mycetome with piece from Terramycin disc (estimated at 2.123 microgrammes in drop of 0.07 ml volume).	
5/1/54	0/30	A mycetome from an oryza larva placed in Earle's solution containing tetrazolium salt. Sealed off by vaseline as usual. No significant change noted until 11/1/54 when the preparation began turning pink. 12/1/54 A few organisms have stained deep purple. Many others are visible only under phase. Few of the stained organisms are typical.	21/1/54
5/2/54	0/30	Granarius egg sterilised externally and then placed in a drop of Earle's Solution and sealed off. A few quite typical organisms. These were in poor condition on 22/2/54.	3/5/54

APPENDIX II

Several slides were made by method C (see page 35) using a small patch of agar instead of a drop of a more fluid medium. The following is an account of the best of these.

28/7/54 — A mycetome from a granarius larva was mounted on a small patch of bacto Difco Nutrient agar on a slide. The preparation was sealed over with a coverslip held in place by vaseline. The whole preparation was set up under sterile conditions.

5/8/54 — Many organisms could be seen. Some with evidences of sprouting. Also some appearances of the small bullet-like organisms covering the typical organisms.

9/8/54 — Thread-like organisms showing every sign of sporulation. A careful drawing was made. This is Figure 4. This was regarded as positive proof of sporulation.

10/8/54 — Confirmation of drawing.
A sterile transfer was made: the slide part of this preparation was given a drop of sterile distilled water and a new sterile coverslip - this became MM1. The old coverslip part was given some new sterile agar medium on a sterile slide and sealed off with vaseline - this became MM2.

MM2 was soon seen to have a contaminant presumably from the medium. It was, fortunately, a clearly recognized contaminant.

Later ampoule like bodies could be seen in MM2.

10/3/55 — In MM2 small coccus-like bodies that originated from the typical thread-like bodies are still present. Contaminants easily distinguished.

APPENDIX III

HANGING DROP CULTURES

SPECIAL

One of the hanging drop preparations produced results of such interest that it has seemed proper to give a very detailed account of its history. The observations given below are virtually extracts from my research diary.

Reference 0/18

Prepared on 9 July 1953. A double drop of the kind already described containing a wire piece for mixing. One drop contained a mycetome and nerve tissue piece from an oryza larva. The other drop contained gut from an oryza adult. Both drops were of Earle's solution but contained in addition a small quantity of a Terramycin solution containing 1.5 micro-grammes of Terramycin per litre. Side A contained mycetome and nerve; side B contained gut element.

11 July 53 — Side A: much activity with millions of organisms; suspect some dividing.
Side B: mostly quiet. Coeca cells are retracting from basement membrane. A few strange bullet-like organisms can be seen - no usual organisms.

14 July 53 — Side A still going well; in some areas chains and rosette like clumps are developing. Both sides had many small darting granules.

Drops were mixed. Standard organisms began to flow over from A to B.

15 July 1954 — Many standard organisms, many of the small bullet kind, some fat "bullets" moving actively; thin chains of beads obviously the origin of the bullets; some thick strands now beginning to break into fat bullets?

16 July 53 — Enough evidence to convince that all kinds are stages of one organism. The drop was beginning to dry out and was supplied with fresh sterile water.

17 July 53 — All stages present. Some of the standard organisms were seen in active wiggling motion and also dividing. The gut piece is degenerating, but is full of bullet-like organisms.

21 July 53 — Transferred to ordinary slides

30 July 53 — Many organisms have black granules. It was discovered that there were a great number of active organisms lower in the drop and requiring a deeper focus to be seen. At one end of this lower part of the drop are standard organisms, at the other end small "dots". By inverting the slide it was possible to see the organisms in the lower part. Both standard organisms and the bullet like organisms were visible in considerable numbers.

11 Aug 53 — Much activity in nerve remnant.

9 Sept 53 — Reproduction seems definite at this time.

20 Nov 53 — No organisms could be seen. Preparation terminated.

APPENDIX IV

Locke's Solution

Sodium chloride	0.9 %
Calcium chloride	0.025%
Potassium chloride	0.042%
Sodium bicarbonate	0.02 %
Dextrose	0.25 %
Peptone	0.2 %

Locke's solution was recommended by Glasser (1917), and the formula is given in his paper in Psyche, 24: 1.

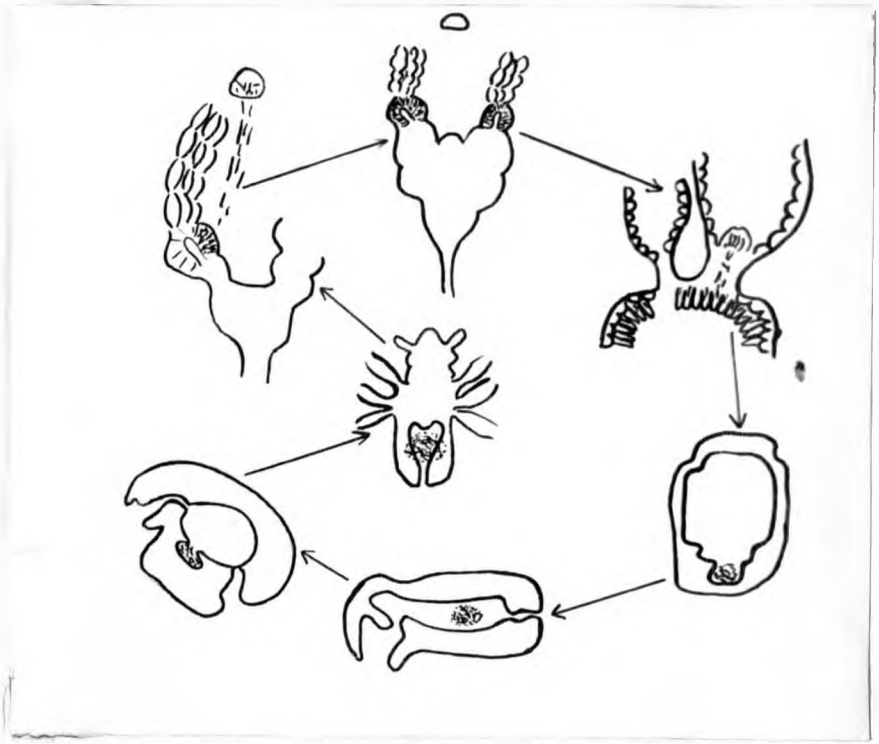

FIGURE 1.
To show the locations, within the louse, of the mycetomal micro-organisms at different stages of the insect's life cycle. Diagrammatic. Adapted slightly from a figure by Reis, 1930.

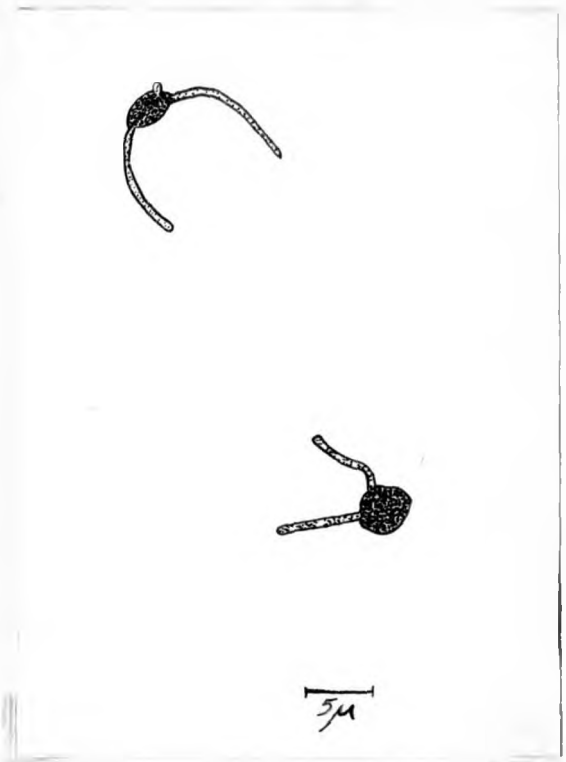

FIGURE 2

Spore germination of mycetomal micro-organisms of Sitophilus granarius. The scale drawing shows two spores, each producing two germ tubes.

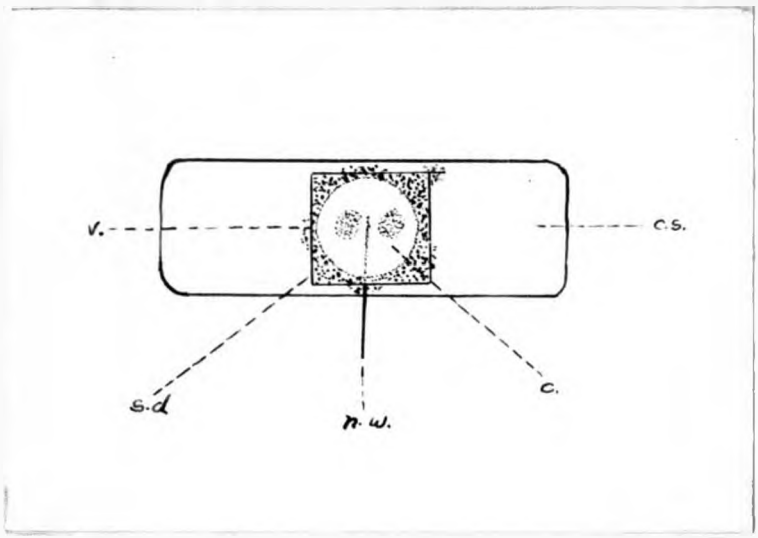

FIGURE 3

Diagram of the arrangement for mixing two sterile drops; c., cell; c.s., celled slide; n.w., nichrome wire mixer; s.d., cover glass roofing over cell and carrying two drops; v., vaseline.

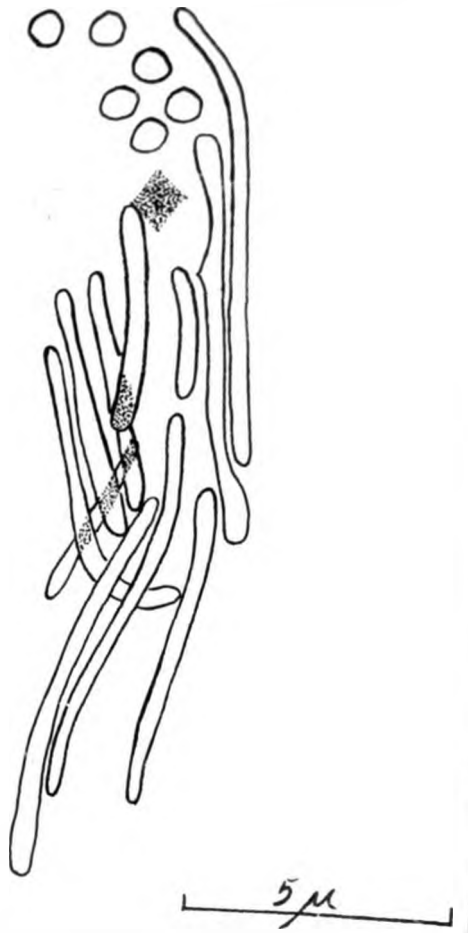

FIGURE 4

Mycetomal micro-organisms of Sitophilus Granarius. One individual has been sporulating by proliferation. Scale drawing from life.

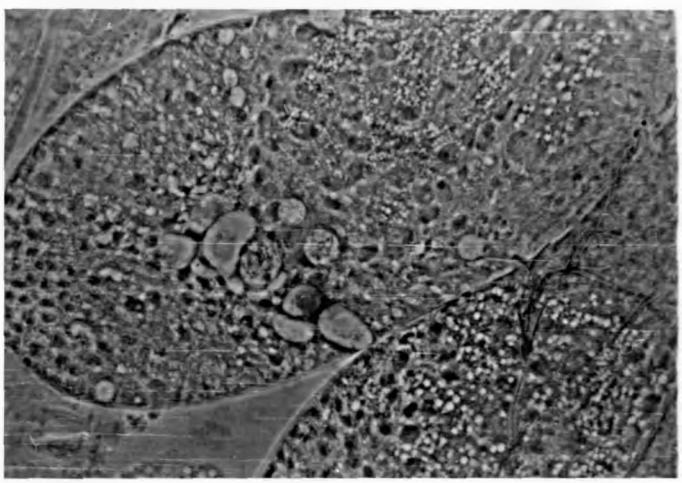

FIGURE 5

Shows parts of three cocca from the mid-gut of S. oryza, showing mycetocytes. Phase; magnification: approx., x 250

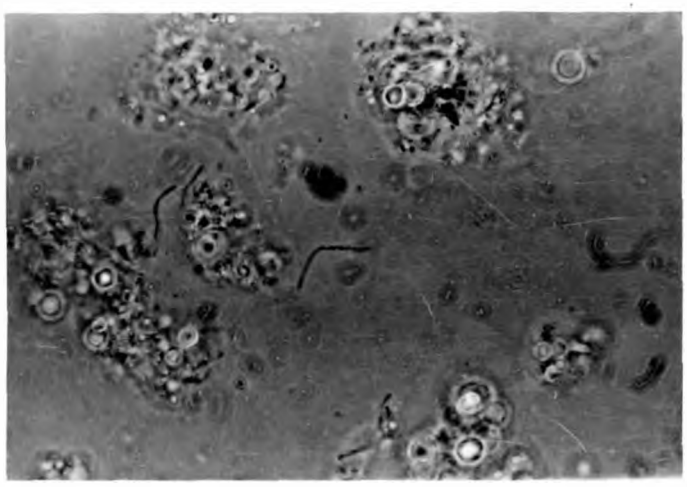

FIGURE 6

Mycetomal micro-organisms in a smear of the ovary of S. granarius. Phase; magnification: approx., x 800.

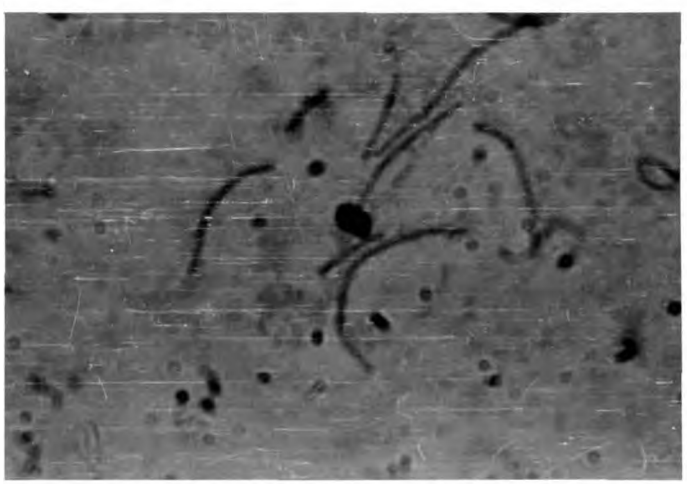

FIGURE 7

Mycetomal micro-organisms from S. granarius stained in Delafield's Haematoxylin, to show size of typical micro-organisms.
Magnification: approx. x 1500.

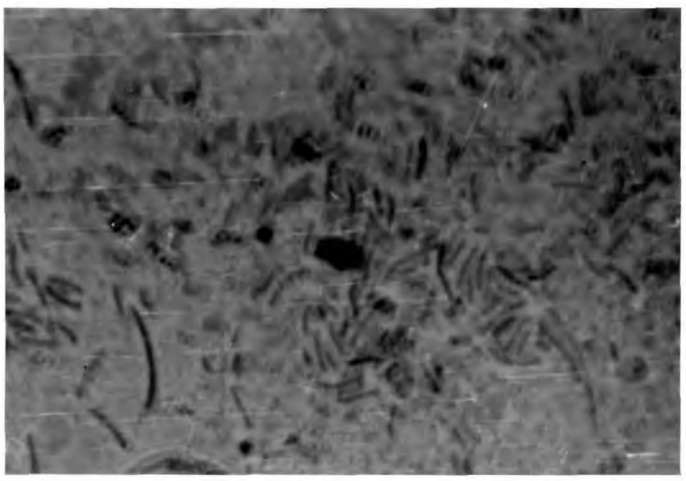

FIGURE 8

Mycetomal micro-organisms from S. oryza stained in Delafield's Haematoxylin to show size of typical micro-organisms.
Magnification: approx., x1500.