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FUNGOUS DISEASES OF THE HONEYBEE
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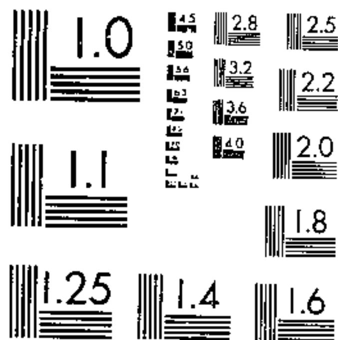

MICROCOPY RESOLUTION TEST CHART
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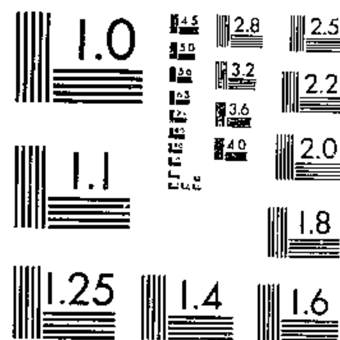

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

TECHNICAL BULL. No. 149 JANUARY, 1930

UNITED STATES DEPARTMENT OF AGRICULTURE
WASHINGTON, D. C.

FUNGOUS DISEASES OF THE HONEYBEE[1]

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INTRODUCTION

The recognition and control of bee diseases is of prime importance in the commercial production of honey. The serious bacterial diseases of the brood have been extensively studied, and the life histories of their causative organisms and the methods for control are fairly well understood. There exist, however, other diseases of the brood and of adult bees, seemingly of lesser consequence, the causes of which are not yet known and for which satisfactory methods of control have not been determined.

The writer has shown in an earlier paper (*5*)[2] that a considerable number of species of fungi occur regularly on adult bees, and less frequently on their brood, which completely mummify all of the softer tissues. Most of these species of fungi are capable of establishing themselves on the brood combs under conditions that often pre-

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[2] Reference is made by italic numbers in parentheses to "Literature cited," p. 40.

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vail within the hive during late winter and spring. The ecology of these fungi on bees in all stages and their relation to bee diseases have never been fully worked out.

During the past three years a mycological study has been made by the writer, in culture and on bees, of a considerable number of species of fungi that were isolated from adults, larvae, and combs. Experiments have been performed to determine whether the fungi that are commonly found on bees are purely saprophytic or whether, under conditions favorable for infection, they can attack and kill healthy bees or brood.

HISTORICAL

The existence of diseases of bees was first recorded by writers before the beginning of the Christian era, but the descriptions are too meager to identify them. The more careful study in Europe of diseases of brood dates from the work of Schirach (*21*) in 1771, but for more than a century following it was quite generally believed that there was only one such disorder. Succeeding reports in Europe supported this view. During the decade just preceding the twentieth century American beekeepers came to believe that more than one disease of the brood existed, and that these diseases were of decidedly different characters. This has been conclusively proven in the United States, chiefly by the investigations of White, who describes and figures two bacterial brood diseases, a filterable virus disease, and one protozoan disease of adult bees, in a series of papers dating from 1917 to 1920. The diseases described by him are sacbrood (*28*), Nosema disease (*29*), American foulbrood (*30*), and European foulbrood (*31*). More recent valuable additions to the knowledge of brood diseases and their control have appeared in the publications of Sturtevant (*22, 23*), and Zander (*32, 33*).

The study of diseases of bees due to fungi has been much later in its development than the study of those caused by bacteria. Much the greater share of the attention of investigators of bee diseases has been given to the two serious bacterial brood diseases, American foulbrood and European foulbrood, caused respectively by *Bacillus larvae* and *B. pluton*. In America only two reports are on record of diseases of bees and their brood caused by hyphomycetous fungi. In 1896 Howard (*14*), of Texas, described a new brood disease which he called "pickled brood or white fungus," caused by a species of Aspergillus to which he gave the name "*Aspergillus pollini.*" Two years later he described (*15*) the same disease as occurring in both pupae and adult bees and stated his belief that this disease had been mistakenly diagnosed by beekeepers as paralysis.

In both reports Howard gives descriptions and illustrations of the disease, which he called "pickled brood or white fungus," which are more readily applicable to the disease known as sacbrood than to those caused by fungi. He ascribed this disease to the pathogenic Aspergillus, *A. pollini.* The dead larvae are described as at first white and watery, later becoming black and swollen, and finally drying down to black scales. In no case was the fungus, which Howard assumes to be the cause of the disease, observed on the larvae, and isolations of the organism were not made. In adult bees the disease is described as causing black, shiny, apparently frozen abdomens.

The affected bees become much weakened, are capable of only feeble, trembling movements, and finally die. As in the case of the brood killed by the "pickled brood" disease, the fungus was not observed on or within the tissues of the infected bees, but when cultures were prepared from the alimentary canals of these bees, *A. pollini* developed constantly to the exclusion of all other fungi.

It appears probable, therefore, that Howard may have observed one or more species of Aspergillus on brood combs and succeeded in culturing these, or other species, from the alimentary canals of adult bees. His technical descriptions of *Aspergillus pollini* are entirely too meager to make it possible to determine which of the numerous species of Aspergillus were observed. If they are pathogenic for bees, it is evident that he either did not observe a true mycosis of bees or confused the condition with other disturbances.

Diseases of bees caused by fungi have not been reported from North America since then. This may probably be attributed to the fact that fungous diseases of bees appear less destructive than the common bacterial diseases of bees and seldom become epidemic.

In Europe, on the other hand, two species of fungi, *Aspergillus flavus* Link and *Pericystis apis* Maassen, are widely recognized as the causative organisms of diseases of brood and adult bees. Of these, *A. flavus* is considered of the greater economic importance, since it attacks worker brood and adult bees, whereas *P. apis* usually attacks only drone brood. The two brood diseases stone brood (Steinbrut), caused by *A. flavus*, and chalk brood (Kalkbrut), caused by *P. apis*, have received more attention from European beekeepers and investigators than has the disease of adult bees caused by *A. flavus*. This might readily be expected as a direct result of the nature of the diseases and the reaction of infected adult bees. Pericystis mycosis is mentioned by Claussen (*8*), Bahr (*2*), and Morgenthaler, Kessling, and Hunselmann (in communications with Claussen). Claussen describes it as benign and transient rather than malignant, affecting capped as well as uncapped drone brood and passing over to worker brood in severe or exceptional cases. He states that dead and diseased larvae may be thrown out of the hive by the bees or allowed to remain in the brood combs, where they become mummified after they are overgrown with white mycelium. The bees, however, usually allow any brood killed by *A. flavus* to remain in the combs for a considerable length of time, or at most only partially remove it, since destruction of the cell walls is often necessary for complete removal.

Slight infection among the brood quickly attracts the attention of an observing beekeeper, whereas he may completely overlook a considerable number of adult bees dead of this disease, owing to the fact that during the active season worn-out field bees die normally in considerable numbers about the hive. The writer, therefore, believes that the importance of Aspergillus mycosis as a brood disease may have been overestimated in comparison with its importance as a disease of adult bees.

Recent research in Europe seems to indicate that other fungi than the foregoing may under favorable conditions infect and kill bees and their brood. Fielitz (*10*), working with three fungi, *Trichoderma lignorum* Tode, *Mucor mucedo* Linné, and *Penicillium glaucum*

Link, found on mummified bees in Germany, was able to infect capped and uncapped brood and adult bees by artificial inoculation with the first two. *T. lignorum* was shown to be capable of becoming actively pathogenic when introduced into healthy colonies on brood combs. When similarly introduced, *M. mucedo* attacked and killed brood in capped cells and an occasional adult bee. In his experiments with *P. glaucum* neither the bees nor their brood were attacked, although it is one of the commonest organisms in the hive.

While making anatomical studies of honeybees affected with constipation, Lardinois (*17*) claims to have observed *Saccharomyces apiculatus* (Reess) Hansen constantly associated with lesions in the tissues. The same organism occurred in the intestines of dead pupae that were thrown out of the hive, in the food of larvae, and in honey. Lardinois asserts his belief that *S. apiculatus* is the sole cause of May disease and that disturbances commonly recognized as constipation, paralysis, convulsions, staggering, malformation, and death of brood are, in reality, all forms of the disease which he called saccharomycosis. He does not support these conclusions by inoculation experiments but draws them solely from the fact that this yeast occurs in "lesions" and in the intestine of bees affected with "May disease." Doctor Lardinois believes, however, that these conclusions may be easily verified.

One is quickly led to believe that bee diseases caused by pathogenic fungi may be more widespread than is commonly supposed. A comparison of conditions in Europe, where a number of recognized fungi are known to cause diseases of bees, with conditions in this country would make it seem likely that the same fungi are capable of attacking bees here. The publications of Thom and Church (*24; 25, p. 200*) show that a great number of strains of *Aspergillus flavus* are found in America. *Mucor mucedo* and *Trichoderma lignorum* also occur elsewhere than in Europe. *Pericystis apis* and *P. alvei* Betts, which, according to Claussen (*8*), differ in certain morphological characters and in their ability to attack brood of bees, have never been reported from North America. Forms of *Saccharomyces apiculatus*, which Lardinois has stated cause disease of bees in France, are widely distributed in North America. The writer has frequently found these forms in North America within the alimentary canal of bees and in honey.

INVESTIGATIONS WITH PATHOGENS

DESCRIPTIONS AND LOCATIONS OF APIARIES USED

Apiaries located in the vicinity of Bronson, Mich., were used in these investigations for securing specimens of fungi and for making tests for pathogenicity and control. These apiaries are situated on boulder clay and sandy drift formations in a general farming community. Three honey flows occur annually in the region, providing a plentiful supply of stores at all times when weather conditions are favorable.

The experimental apiary of the botanical laboratory of the University of Michigan at Ann Arbor was also used in these investigations. This apiary was located on the roof of the Natural Science Building, thus affording excellent conditions for recovering diseased

bees. With the beginning of cold weather the colonies were placed in an attic where a temperature of about 55° F. was maintained, affording facilities for manipulating the bees during cold weather without danger of chilling them. The general conditions here with respect to honey flows and weather factors are essentially the same as at Bronson, excepting that here the early spring honey flow is somewhat heavier on account of the surrounding fruit farms. On the other hand, the fall honey flow from wild flowers is somewhat less.

Two apiaries of the bee-culture laboratory of the Bureau of Entomology at Somerset, Md., were used during the summers of 1924, 1925, and 1926. One of these was located at the laboratory, while the other was about a quarter of a mile away. Forest trees, particularly species of Acer and Salix, provide a source of nectar during early spring, but the main honey flow is from the tulip tree (*Liriodendron, tulipifera*). Occasionally basswood (Tilia), spruce (Picea), and locust (*Robinia pseudoacacia*) yield appreciable quantities of nectar. The fall flow here is light and of short duration.

RACES OF BEES

The bees used for the tests for susceptibility were for the most part Italians and Italian hybrids, including workers, drones, and queens. No other races were used for inoculation experiments. Pathogenic organisms were isolated, however, from Carniolan bees.

The colonies used for inoculation experiments were, most of them, in 5-frame hives (nuclei) containing three or more frames of brood. Normal colonies in standard 10-frame hives were also occasionally used.

THE FUNGI STUDIED

The fungi used were isolated from bees collected from widely different sources.

The greater number of forms were from bees from the experimental apiaries. The first isolations of fungi were made from dead bees and their brood collected at Bronson, Mich., during the early brood-rearing season of 1924, and others were made from bees collected at this same apiary during the spring of 1925 and that of 1926. During the summers of the three years that this work was in progress fungi were collected from the apiaries of the bee-culture laboratory of the Bureau of Entomology. Among the forms obtained here were duplicates of most of those collected at Bronson and elsewhere. Important collections were also made from the experimental apiary at the University of Michigan during the academic years of 1924–25 and 1925–26.

In addition to the collections from the experimental apiaries, fungi have been isolated from samples of bees or brood sent to the bee-culture laboratory from all of the important beekeeping regions of the United States. Although the forms isolated from such samples duplicated those obtained from the experimental apiaries, their presence gave an indication of the extent of the range of these forms. All of the Aspergilli in the following list appeared with sufficient regularity to indicate their distribution among bees in all parts of the United States, and two species, *A. flavus* and *A. fumigatus*, were isolated on several occasions from bees imported from Europe. The

yeasts were also isolated from bees from numerous sources within the United States. *Trichoderma koningi* was isolated from adult bees in Maryland and from mummified larvae from Oregon. *Pericystis apis* was also isolated from mummified brood sent from England and from Germany.

The fungi included in the following list were found to attack and kill bees when the latter were inoculated experimentally. Among the Aspergilli, with the exception of *Aspergillus oryzae* and *A. parasiticus*, many cultures other than those indicated were isolated from bees and tested for parasitic properties. This is true especially of the *A. flavus-oryzae* and the *A. fumigatus* groups.

Aspergillus flavus (Link), sensu Thom and Church. Author's collection: 1, 3, 4, 5, 7, 9, 12, 28, 12340. Thom and Church collection: Ao5c, 183, 108.

Aspergillus oryzae, (Ahlburg, Cohn), sensu Thom and Church. Thom and Church collection: 113 L, Ao5b.

Aspergillus effusus, VIII; D. C.

Aspergillus parasiticus Speare.

Aspergillus flavus-oryzae, sensu Thom and Church. Thom and Church collection: Aop, Apb, Aob, Ao5a.

Aspergillus fumigatus (Fresenius), sensu Thom and Church. Author's collection: 1, 33, 12287, 12288. Thom and Church collection: Yates IV, 118. 4063–c–18.

Aspergillus nidulans (Eidam), sensu Thom and Church. Author's collection: 1. Thom and Church collection: 110, 4010.4, 4415.

Aspergillus ochraceus (Wilhelm), sensu Thom and Church. Author's collection: Conn.; D. C. Thom and Church collection: 112, 2309, 4020.4, 4065.1, 4640.476, 4640.483.

Aspergillus glaucus (Link), sensu Thom and Church. Author's collection: 1, Ann Arbor, Mich. Thom and Church collection: 3528.7.

Saccharomyces ellipsoideus Hansen.

Saccharomyces cerevisiae Hansen.

Saccharomyces apiculatus (Reess), Hansen.

Mycoderma cerevisiae (Desm), Hansen. Author's collection: I, II.

Torula sp.

Mucor hiemalis Wehmer.

COLLECTION OF FUNGI FROM COLONIES AND FROM INDIVIDUAL BEES

Fungous pathogens of honeybees were collected from colonies and from individual bees in the vicinity of experimental apiaries. Early in the spring the interior of the hives and the ground about the apiary were examined for infected bees. Similar examinations were continued during the summer and fall, and less frequently during the winter in colonies wintered indoors. Adult bees, including queens and drones, with fungus-infected tissues or with conidiophores and conidia upon their outer surface yielded pathogenic forms. Crawling bees, unable to fly and apparently diseased, were collected. These bees were either used immediately for isolation experiments or were caged, under conditions favorable for the continued development of fungi, for examination later. Usually the greater number of such bees died within a short time after having been caged. At other times their condition appeared not to shorten their lives. The number of infected bees appeared to increase noticeably after warm summer rains and those collected yielded a higher percentage of pathogenic organisms. Fungus-infected larvae were found in weak colonies in early spring. Infected larvae were also found in colonies in which facultative parasites were growing on the brood combs. It

was not uncommon to find brood attacked during the spring in colonies which, owing to weather conditions or other factors, had a high mortality when normally the population should have been increasing. The quantity of diseased brood was found to be less during dry weather. At all times of the year strong colonies were found to be relatively free from pathogenic fungi.

EXAMINATION OF DISEASED BEES AND ISOLATION OF PATHOGENIC FUNGI

The methods employed in the isolation of pathogenic fungi from bees must depend to a considerable degree upon the species of infecting organisms and the extent and state of their development. Samples of dead bees or bees in various stages of development of the disease were therefore examined microscopically to determine the stage of growth of the fungus. Isolation of filamentous pathogens was least difficult when spores practically free from contaminating organisms were present upon the body of the bees. The tip of an inoculating needle drawn to a sharp point was moistened by dipping it into sterile agar. The tip of a spore-bearing branch with mature spores was lightly touched and a few spores transferred to an agar plate. Pure cultures of the organism desired were often obtained by this method without additional effort. When more than one fungus was fruiting upon the body of the bee or even when scattered spores of other fungi were present, this method usually resulted in mixed cultures. Pure cultures of the Aspergilli, which are mostly rapid growers, were often obtained from mixed cultures by cutting off the tip of a young mycelium and transferring it with a small quantity of agar to a fresh plate. When contamination with other fungi was heavy, or when the contaminating organisms grew more rapidly than the fungus to be isolated, the dilution-spray method devised by Kauffman (*16, p. 364*) was employed with good results. A suspension of conidia was first made in a tube of sterile tap water. This was diluted until droplets blown from a glass tube drawn to an extremely fine bore at the tip contained an average of about one spore. One or two plates of nutrient agar were sprayed with this suspension, the spray being so regulated that droplets on the agar remained separated by a distance of about 3 millimeters. When germinating spores first appeared single spores with germ tubes were cut and transferred with small pieces of agar to separate poured plates. Two or more such cultures were prepared in each instance. When contamination occurred transfers were made of the few hyphae from the single spore mycelium as soon as the contaminating organism became visible.

When spores were present only within the exoskeleton of the bees, and especially when within the alimentary canal, isolation of pure cultures was difficult on account of the presence of great numbers of bacteria. When these conditions were met with, the acidity of the nutrient agar upon which the suspension of spores was sprayed was made slightly greater with tartaric acid, and the cultures were kept at a temperature of about 10° C. until the spores had germinated. Though the growth of the fungus was retarded when kept at this temperature, the growth of bacteria was practically inhibited. Single germinating spores were then cut out along with any adhering

bacteria and transferred to sterile plates. These cultures were kept in the cold room until fungus mycelium had grown to a distance of several millimeters beyond the zone of bacterial growth. The tips of hyphae, apparently free of bacteria, were then cut off and transferred to sterile plates. This process usually resulted in pure cultures. Whenever bacteria appeared, the process was repeated as often as was necessary.

A method modified somewhat from the foregoing was used when only mycelium was present within the tissues of the bees. Small pieces of tissue containing mycelium were placed on plates of nutrient agar and kept at 10° C. It was not always necessary to place these cultures in the cold room when they could be closely watched and isolations made from the first hyphae that grew into the agar.

Hyphae grew out rapidly from the infected tissue, and the tips of the first appearing hyphae were cut off and transferred before bacteria or mycelium from spores could spread beyond the point of inoculation. Pure cultures were made from these after spores had matured.

The isolation of yeasts from the alimentary canal of the bee required a more delicate technic than the isolation of filamentous fungi because of the presence of other contaminating forms. When yeasts occurred within the tissues or blood of bees they were readily isolated since they were usually found here in pure or nearly pure culture. In the isolation of yeasts a sufficient quantity of acid was added to the medium to retard the growth of bacteria, thus facilitating the isolation of pure yeast cultures.

To obtain pure yeast cultures the spray method described above was used. A suspension of yeast cells was sprayed on beer-wort agar or on Leonian's agar in Petri dishes. The location of the droplets after evaporation was marked by a perceptible deposit of lime from the tap water. After from 12 to 24 hours these spots were examined under the microscope, and those that contained yeast colonies developed from single yeast cells were marked by scratching the agar about them with a sterile needle. When these colonies had developed to contain several hundred cells second dilutions and sprays were made from them. From these cultures, or from similar succeeding ones when bacterial contamination was heavy, yeast colonies free from bacteria were obtained. Colonies, the origin of which could be traced to a single cell, were chosen for isolation a second time to avoid the possibility of mixed yeast cultures. When the original culture was obtained from the rectum or ventriculus of a bee contamination with bacteria was usually heavy, and several repetitions of the process were often necessary to obtain bacteria-free cultures.

CULTURE METHODS

For the culture of the organisms studied the usual mycological equipment was used.

Erlenmeyer flasks of 500 and 1,000 cubic centimeter capacity, filled to a depth of about 3 centimeters with liquid nutrient media, were used for the culture of pathogenic organisms in experiments to determine the production of metabolic toxins. Flask cultures iden-

tical with these were also prepared to inoculate large quantities of nutrient solution to be used in the preparation of brood-comb cultures.

PREPARATION OF THE CULTURES

All of the fungi tested and found to be pathogenic for bees were cultured on one or more of the solid or liquid nutrient media used. Cultures of these fungi were prepared by inoculating the nutrient medium in the culture dish with a single spore or with spores or mycelium from pure cultures. Normal room temperatures were generally used although cold rooms and electric ovens were employed at times. Combs were sterilized in a 20 per cent aqueous solution of formaldehyde. Two liters of nutrient solution containing spores of the desired organism taken from a pure culture were poured into each comb. These combs were kept in a sterile moist chamber until spores had matured over the greater part of their surface. Practically pure cultures were obtained by this method. The few contaminating spores which settled on the combs while the spore suspension was being poured were soon overgrown and rarely matured.

It was found better to keep the moist chambers out of doors while the fungi on the combs were developing, or at least in rooms where beekeeping equipment, particularly extracting and brood combs, were not stored, since under proper atmospheric conditions the combs are overgrown with fungi, principally Penicillia and Aspergilli. An abundance of spores of such pathogenic organisms within the room will add to the danger of spreading the molds to other combs.

One strain of *Aspergillus ochraceus* was found to produce but slight growth on liquid media. Cultures of this organism on combs produced few or no spores and were usually soon overrun by species of Penicillium. Inoculation of colonies with this organism were generally unsuccessful. This difficulty was overcome by culturing the organism on heavy sheets of reinforced blotting paper. The blotting paper was first sterilized and then saturated with nutrient agar before inoculation by spraying with a suspension of spores. After a crop of spores had matured the sheets of blotting paper were suspended in the hive containing the colony to be inoculated.

THE MEDIA EMPLOYED

Several nutrient media, both synthetic and natural, were used during this work for the artificial culture of the organisms. Solid media which were used extensively for isolation, study, storage, and shipping purposes, were prepared by the addition of agar, and less frequently gelatin, to the nutrient solutions. The agar and gelatin were omitted in preparing cultures for inoculation purposes when it was desired to have a large quantity of spores that could be readily freed from the medium. Liquid nutrient media were also used when the object was to extract toxic products of pathogenic organisms, and for the culture and study of yeasts. Natural media, such as potato, carrot, and milk were rarely used. Occasionally liquid media to which a small quantity of tartaric acid was added were used when it was desired to eliminate bacteria from the yeast cultures. The media used in the course of these investigations are described on the pages that follow.

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MALTOSE AGAR

Maltose	grams	5.00
Magnesium sulphate	do	.10
Calcium nitrate	do	.50
Potassium phosphate, dihydrogen	do	.25
Agar	do	15.00
Water	liter	1

Maltose agar, prepared according to the foregoing formula, which is the standard culture medium in use in the cryptogamic laboratory at Ann Arbor, Mich., was used for isolation and culture of filamentous fungi and for isolation of yeasts. This agar remains clear and light colored and may be autoclaved at 15 pounds for 20 minutes, or even at higher temperatures and for longer periods, without hydrolysis sufficient to prevent solidification.

Single germinating spores are easily located when sprays are made on maltose agar, and transfers are facilitated on account of its firmness. Most of the pathogenic filamentous fungi used in these investigations grow well on it and produce spores abundantly. The yeasts grow slowly, and for this reason it serves well for their isolation, but it is unsatisfactory for culturing, and for the study of these organisms.

LEONIAN'S NUTRIENT AGAR

Potassium phosphate, dihydrogen	grams	1.25
Magnesium sulphate	do	.625
Peptone	do	.625
Maltose	do	6.250
Malt extract	do	6.250
Agar	do	13.00
Water	liter	1

Leonian's agar is darker and softer than maltose agar and is therefore less desirable for the isolation of single germinated spores. On account of its softness, cultures do not ship well on it unless partially dried. It was found to give the best results for the isolation of filamentous pathogenic fungi from the infected tissues of bees. Hyphae from small pieces of infected tissue spread more rapidly into this agar than into peptone agar; consequently the danger of contamination from germinating spores of fungi other than the one desired is lessened, since spores appear to germinate with equal rapidity on either of these agars. Conidia of the filamentous fungi form early and abundantly on Leonian's agar, and all of the yeasts encountered in these investigations grew well on it.

CZAPEK'S SOLUTION AGAR

Sodium nitrate	grams	3.00
Potassium phosphate	do	1.00
Magnesium sulphate	do	.50
Potassium chloride	do	.50
Ferrous sulphate	do	.01
Sucrose	do	30.00
Agar	do	15.00
Water	liter	1

All of the Aspergilli considered here and described by Thom and Church (*25*) were grown by them upon this medium. It was therefore used for the culture and identification of all of the forms of Aspergilli that have been found to be pathogenic to honeybees.

Isolations of single spores can be made as readily from Czapek's solution agar as from maltose agar, consequently it was frequently used for this purpose.

Beer-wort Gelatin

For the study of yeasts, beer wort was prepared from malted barley and hops as follows: Two hundred grams of the finely ground barley was soaked for an hour, with occasional stirring, in a liter of distilled water at 60° C. Four grams of hops was then added and the mixture boiled for an hour during which time it was stirred at intervals of a few minutes. Water was added from time to time to maintain the original volume. The barley meal was then separated from the liquid by straining it through closely woven cloth, and the liquid was cleared with the white of eggs. The quantity of maltose present was determined, and water was added until the liquid beer wort contained approximately 3 per cent of this sugar. Gelatin was added to the clear beer wort when a solid medium was desired.

Potato Media

Culture media for yeasts were prepared by cutting pieces of potatoes the proper size for the culture dish and sterilizing them after stoppering or covering. Potato-broth agar was prepared at first for the purpose of storing yeasts, but since there was no particular advantage in its use, it was replaced with beer-wort gelatin.

Liquid Nutrient Media

Synthetic and natural liquid nutrient media were prepared according to the formulas just given under solid media, except that agar and gelatin were omitted. Honey, dextrose, and levulose were sometimes substituted for the sugars given in these formulas. Beer wort was used extensively in the study and identification of yeasts, since most of the morphological and cultural characteristics of yeasts reported in the past were made from growth in this nutrient medium.

Sucrose, dextrose, levulose, maltose, galactose, dextrin, inulin, raffinose, d-mannose, and l-arabinose were tested in liquid media to determine the ability of the yeasts listed on page 6 to ferment these sugars.

EXPERIMENTS WITH BEES

PRELIMINARY INOCULATION WITH MOLDED COMBS

In a previous study of the fungi associated with honeybees (*5, p. 63, 68*), it was observed that species of two groups, the Penicillia and the Aspergilli, occur with greater frequency than do other forms. These fungi, Penicillia and Aspergilli, were found within the bodies of bees where the mycelium had permeated all of the softer tissues, resulting in a characteristic mumification. Formation of spores of these fungi was observed to occur both within the exoskeleton and upon the exterior. After these observations a question arose as to the relation of these organisms to the honeybee. Was the bee attacked before death, or afterwards? If before, death might have resulted from infection by the fungus.

To determine this point preliminary experiments were conducted in the writer's apiary at Bronson, Mich., during the spring of 1924. A search was made of the entire apiary, and the combs showing the best growth of mycelium and spores of Penicillia and Aspergilli were selected. Both brood combs and extracting combs were found to contain these forms. Brood combs overgrown with Penicillia were readily found in colonies that had wintered badly or had died during the winter. Combs even partially covered with Aspergilli were less abundant. In order to find a sufficient number of combs containing Aspergilli, it was necessary to examine not only the brood combs but the extracting combs as well.

The combs containing the fungi to be tested for pathogenicity were placed in weak, but apparently healthy, colonies. As a result of these inoculations a varying degree of loss of bees was noticeable, and in one case the death of the entire colony resulted. The infected bees appeared to die at a considerable distance from their hives, and dead bees were not found in unusual numbers on the bottom board or near the hives. Numerous bees unable to fly were found crawling about the apiary during the time the inoculation experiments were in progress. It was found that about 50 per cent of the dead bees collected from the fronts of the colonies that had been given infected combs produced conidiophores of apparently the same species as those used in the inoculations. In order to promote the production of conidia the dead bees were kept in moist chambers at room temperature for from 5 to 7 days.

The results obtained in these preliminary experiments were considered sufficient to warrant further study. The experiments which follow were therefore made with pure cultures of a considerable number of fungi suspected of possessing qualities pathogenic to bees.

INOCULATION EXPERIMENTS WITH PURE CULTURES

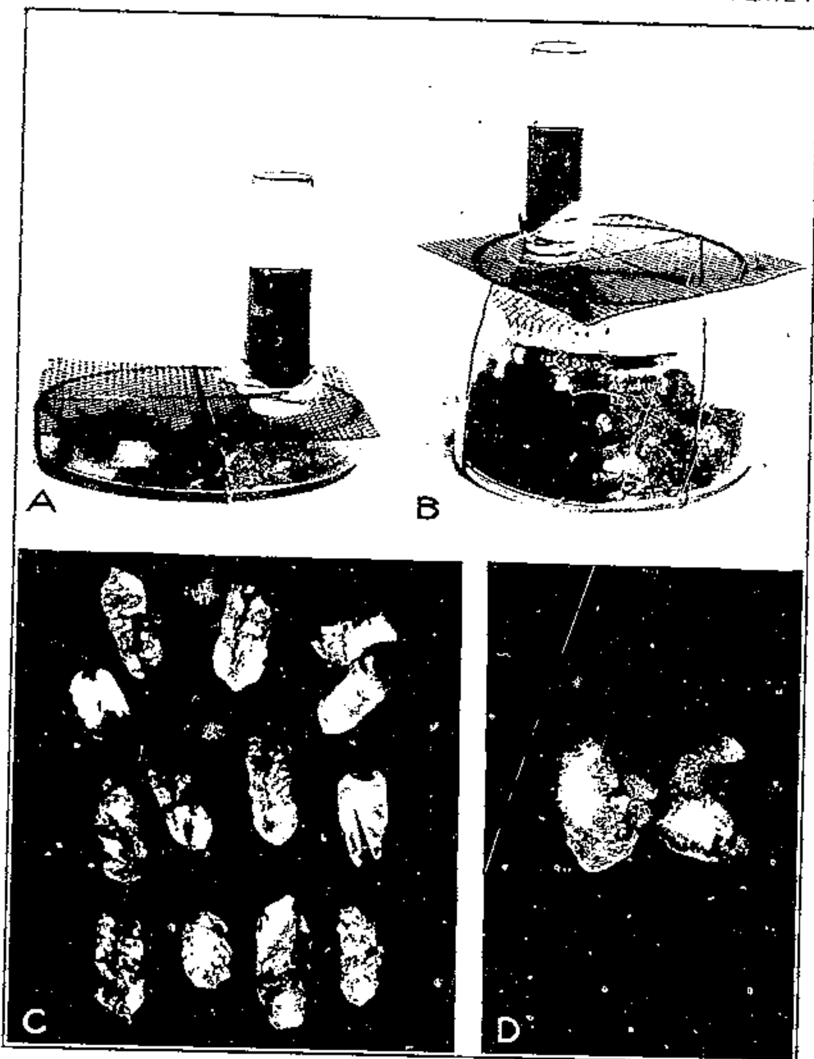
Experiments with pure cultures of the organisms were of two general types. In the one, adult bees were inoculated and kept in cages during the experiment. In the other, large quantities of the inoculum were used on bees in normal colonies.

EXPERIMENTS WITH CAGED BEES

The types of cages used for confining the bees during the experiments are shown in Plate 1, A and B. The cage shown in A was found to be the most convenient, as the removal of individual bees was facilitated. In this cage, also, the food supply was easily reached by the bees.

Some trouble was experienced at first in devising a method of feeding that would furnish the bees an abundant supply of food at all times without danger of daubing them with it. This smearing invariably happened in the use of candy in culture dishes when the nutrient agar was not thoroughly dried, since water was rapidly taken up from the agar by the candy.

A satisfactory method was finally found and used in the succeeding experiments. Small vials were filled with sugar solution or honey and covered with one or more thicknesses of cheesecloth fastened with rubber bands. These were inverted on the screen covers



A and B.—Types of cages used in inoculation experiments with adult bees.
C.—Brood dead of Aspergillus mycosis produced experimentally by inoculation with a culture of *Aspergillus flavus* on a brood comb.
D.—Larvae recently dead of Aspergillus mycosis caused by the penetration of the larval skin by germ tubes of *A. flavus*.

of the cages so that the food could readily be reached by the bees. (Pl. 1, A and B.) The number of thicknesses of cheesecloth required depended upon the density of the sirup used. Heavy honeys required but one thickness, but for dilute sugar sirups two or more thicknesses of cloth were necessary to prevent leakage.

Bees in cages were inoculated by being placed on cultures of the fungi or by mixing the spores with their food. Within the laboratory, where cultures of fungi were kept, it was unsafe to inoculate bees, by the first of the two methods, with fungi that form dry and dustlike spores; for by fanning their wings the bees sent a dust of spores into the air. Consequently, inoculations were made outside of the laboratory. When spores of fungi were mixed with the food given to the bees the danger of contamination of cultures within the laboratory was largely avoided. Abundant space outside of the laboratory being available, however, the first method of inoculation was generally used, since it probably approached nearest to the natural methods by which bees become infected. Complete inoculations resulted from placing the bees for one or more minutes on plate cultures that had matured a good crop of dry spores. After inoculation the bees were either removed to sterile dishes or caged, until the experiment was complete, on the culture upon which the inoculation was made. A cone made by cutting off the smaller end of a funnel, so that a hole with a diameter of about 1½ inches was left at that end, was used for transferring the bees from the cultures to the cages. The small end was placed over the cage, and the culture dish containing the bees was opened quickly and inverted in the mouth of the funnel, completely closing it. When the bees had entered the cage the cone was removed and the screen cover quickly placed in position and fastened with rubber bands. After a little practice this operation of transferring could be successfully performed without injuring the bees, success depending upon speed and precision of manipulation.

As a measure of precaution, when several fungi with dustlike spores were being tested at the same time, inoculations on dried cultures were made out of doors and the cages placed in different rooms, or in different parts of the same room. This prevented, to a large extent, infection with pathogenic fungi from other cages. When this precaution was not observed, a high percentage of infection, at times reaching more than 70 per cent, could be traced directly to the crossing over of spores from adjacent cages. When spores of fungi were mixed with the food given to the bees these precautions were unnecessary.

Selection of Bees for the Experiments

Old bees from the field force of a colony die rapidly when caged, even under conditions of temperature, moisture, etc., that duplicate those of the hive. Their early death when caged appears to be due in part to continued exertion in an effort to escape. Records of the death rate among infected bees are therefore unsatisfactory when field bees are used. Young bees for caging were obtained by setting aside frames of emerging brood a few days before bees were needed, or by lightly shaking frames of bees to rid them of most of the old bees, as young bees cling more tightly to the combs than do the older

ones. Young bees are also more easily handled, since they are less excitable when caged. Young drones and queens, which may or may not have recently emerged, are quite as satisfactory for these experiments as are worker bees, and were frequently included.

EXPERIMENTS WITH COLONIES

Colonies were inoculated by placing matured dry cultures of fungi on brood combs or on strips of blotting paper between the brood combs. One or two of these cultures were put in each of the experimental colonies. Complete infection was obtained somewhat sooner with two comb cultures than with one, though in order to secure complete infection of a colony it was necessary to use cultures with an abundance of dry spores on the greater portion of the surface. Spores were soon spread to all parts of the hive by the worker bees and after three or four days were found in the food of the brood and in the alimentary canal of all the adult bees examined. Colonies were less frequently infected by mixing spores with dilute sugar sirup and confining the bees to the mixture for food.

BLOOD INOCULATION EXPERIMENTS

The blood of the honeybee is well protected from the entrance of microorganisms by the tough exoskeleton. As far as is known, infection by pathogenic organisms occurs only by way of the alimentary canal. Practically nothing has been done to determine the resistance, or lack of resistance, of the blood of the honeybee to infection by microorganisms. A series of blood inoculations of adult bees was therefore made with the fungi used in these investigations.

While they were being inoculated the bees were held between the thumb and the second finger, with the wings turned upward and held with the fore finger. The propodeum was moistened by lightly rubbing it with a wad of cotton or filter paper saturated with a water suspension of the organism under observation until a film was spread over the surface. This portion of the exoskeleton was carefully punctured with a fine-pointed sterilized needle. When the needle was withdrawn the water containing the spores was spread into the wound. As an alternative for the first method, the needle was dipped into the suspension of spores and the tip inserted into the wound a second time.

Checks, to determine the effect of the punctures upon the bees, were prepared by a similar treatment, except that spores were omitted from the water with which the propodeum was moistened or in which the needle was dipped. Inoculations were also made at other points on the body, but fatal injuries resulted more often when inoculations were made in places other than the propodeum. Inoculated and check bees were kept in cages until the experiment was completed.

EXPERIMENTS WITH BROOD

In addition to the inoculation of colonies in which spores reached the food of all uncapped brood, uncapped larvae were inoculated directly from plate or tube cultures. Dried cultures in Petri dishes or tubes were scraped with a scalpel or inoculating needle to loosen

and separate the spores. The dry spores were then shaken only lightly over the brood. If too many spores were shaken into the cells, the worker bees set to work at once to clean them out, and sometimes removed the brood before any infection could be determined. If the inoculated brood is not removed by the workers within 24 hours, it is usually allowed to remain in the comb unless death from infection occurs.

A somewhat surer method was used with fungi that do not produce abundant spores, and when the evidence obtained with dry spores was not considered conclusive. Water suspensions of spores were prepared and small drops added to the larval food with a pipette drawn to a slender tapering point. Larvae were rarely removed from the cells following this treatment except after infection occurred and death had resulted. Close observation, however, was always necessary to determine the exact fate of the inoculated brood. In strong colonies the workers often remove the brood before infection can result if the inoculation is heavy, or they may remove them from the cells, often piecemeal, before symptoms of the disease appear. This method offers quick and easy means, however, of testing the pathogenicity of a large number of organisms for brood of bees.

While examining brood inoculated by the methods just described it was occasionally noticed that tufts of mycelium appeared on the brood before other symptoms of diseases had developed. (Pl. 1, D.) Microscopical examination of such larvae showed that these tufts had developed from mycelium beneath the skin of the larvae. This mycelium was much more abundant in the tissues beneath the tufts than elsewhere. If infection occurred within the alimentary canal one would expect to find mycelium more extensively developed there, at least immediately following infection, than in the tissues just beneath the larval skin. It appeared that the mycelium observed on a few larvae soon after inoculation originated from infection through the skin rather than from within the alimentary canal.

To find whether germ tubes from spores germinating upon the moist skin of larvae can penetrate the skin to the tissues beneath, masses of spores, each about the size of a pinhead, were smeared on a number of larvae in brood combs. The position of these within the comb was marked by removing the brood from the two surrounding rows of cells. The infected larvae were examined twice daily during the three days following. In another method that was used larvae were removed from the comb and kept in watch crystals on a 70 per cent solution of sugar sirup or honey during the experiment. Masses of spores were placed on the larvae in the same manner as if they had been left in the comb. The watch crystals with the larvae were kept between observations in an incubator at 36° C. This method was finally used to the exclusion of the former, for when the larvae were left in the hive, spores placed upon the skins were often removed by the workers or mixed with the food in the cells as the result of movements of the inoculated larvae. This frequently resulted in infection through the alimentary canal. If care was used in placing the moist mass of spores on the skin of the larvae in watch crystals, spores rarely reached the alimentary canal. Larvae move very little when on sirup; consequently the spores remained in the position in which they were placed.

SECONDARILY INFECTED BEES

The accurate determination of the effects of an inoculation upon adult bees demands frequent observations and records. If examination is delayed beyond two days after death, secondary organisms may materially affect the symptoms and obscure the pathogenic organism. Examination during the last stages of disease before death, or immediately after death, furnishes the most conclusive evidence of the pathogenic relations of an organism. The presence of vegetating microorganisms in bees at this time is proof of their growth and multiplication within living bees. As apparently normal adult bees harbor large numbers of certain bacteria and yeasts within the alimentary canal, particularly within the ventriculus and the small and large intestines, their presence is of little pathogenic significance.

On the other hand, much weight may be given to the presence of vegetating organisms of filamentous fungi within the alimentary canal of living bees. The presence of vegetating organisms of any sort within the blood or tissues is evidence of their pathogenic growth, since the tissues and blood of healthy bees are always sterile. Under favorable conditions, however, they may be invaded after death by organisms present within the alimentary canal.

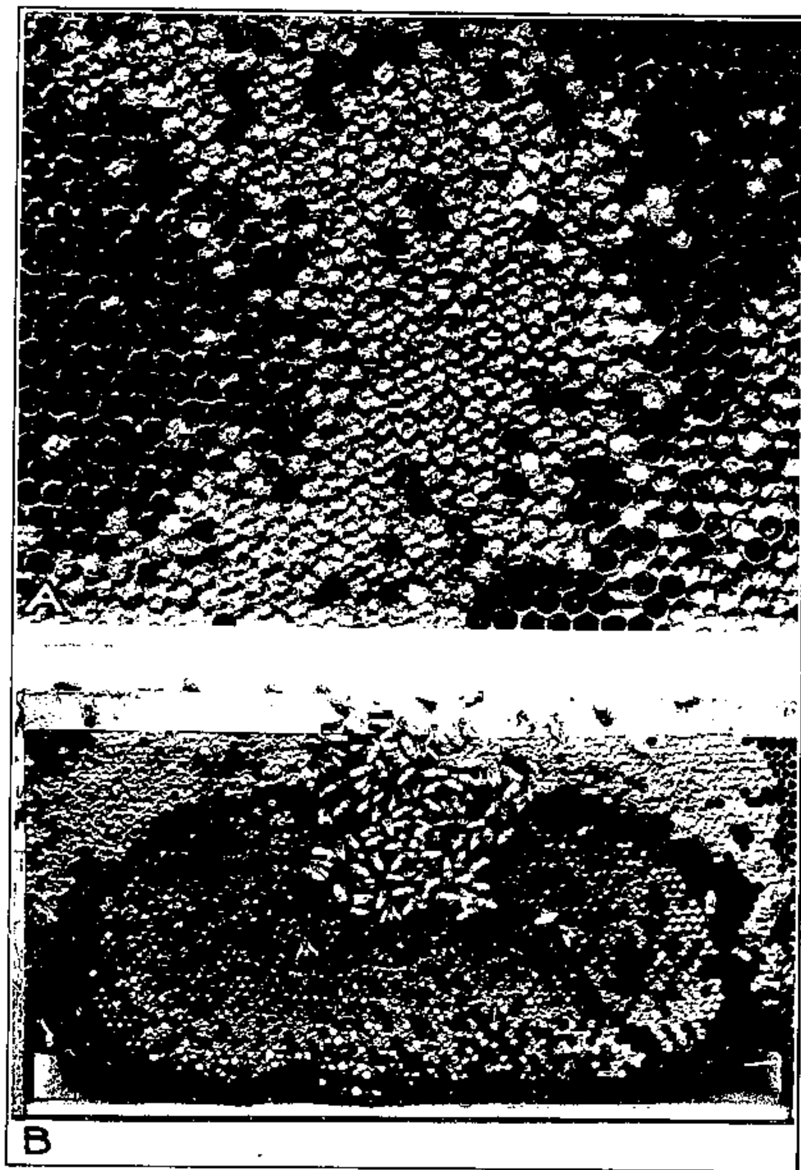
What has been said concerning diseases of adult bees is true to a lesser extent for diseases of their brood. Normally brood harbors fewer bacteria than do the adult bees. During the first two or three days of the life of the larvae, when their food consists only of pap, which is believed to be a glandular secretion, it is often difficult to demonstrate the presence of microorganisms within the digestive tract either microscopically or by culture. When such young larvae die of other causes than bacterial disease the remains frequently dry down without being attacked by putrefactive bacteria or fungi. Microorganisms are found within the alimentary canal of older larvae and pupae, after pollen and honey are mixed with the food, but they are fewer in number than within the worker bees of the field force. Secondary invaders rarely appear after the death of infected brood with the possible exception of those dying from European foulbrood. The causative organism of American foulbrood can be recognized in old brood remains, whereas the absence of putrefactive organisms is the rule with sacbrood.

During these experiments examinations were made at frequent intervals after the inoculations were made. Adult bees were watched more closely than brood in order to observe the symptoms of disease before they were modified by secondary invaders. Records were kept of microscopical and macroscopical examinations and were compared with similar records of bees used as checks upon the experiments.

RESULTS OF INOCULATIONS AND SYMPTOMS OF THE DISEASES PRODUCED

SYMPTOMS PRODUCED BY THE ASPERGILLI

The inoculation of bees with a number of species of Aspergillus resulted in the rapid appearance of mycosis and the death of the bees. With some of the more virulent species all of the bees inoculated and



A.—Brood comb with brood dead of Aspergillus mycosis produced experimentally by inoculation with a culture of *Aspergillus flavus* on a brood comb.
B.—Brood comb with brood and young bees from a colony inoculated experimentally with *A. flavus*, taken after the older bees had deserted the hive before succumbing to Aspergillus mycosis.

kept in cages were attacked and killed within from two to four days. With less virulent species the bees lived somewhat longer and a varying percentage of the inoculated bees escaped infection. At times, with species not ordinarily found on bees, only about 5 or 10 per cent of the inoculated bees died of infection.

The results obtained in colonies were quite similar to those obtained with bees in cages. When colonies were infected with one or two brood-comb cultures of virulent species of Aspergillus, all, or nearly all, of the brood and bees of the colony were killed by the fungi. Illustrations A and B of Plate 2 show brood combs from infected colonies after most of the field bees had died from infection with *Aspergillus flavus*.

With less virulent organisms the death rate for the first day was considerably lower, and at times only part of the population of the colony was killed. In the latter case the activity of the colony returned to normal after the infected combs had been cleaned and the dead brood removed from the brood combs.

When the inoculations were made by placing spores of pathogenic Aspergilli in the blood of bees through needle punctures, disease and death usually resulted. A few larvae were attacked as a result of direct penetration of the skin by germ tubes. (Pl. 1, D.)

Symptoms of Mycosis in Adult Bees

The first noticeable symptoms of infection by Aspergillus in adult bees is their weakening and restlessness, and the continued effort on the part of the sick bees to escape from the cluster of healthy ones. In cages the earliest affected bees can be picked out from among those not yet infected by selecting bees that continue to crawl at a time when the greater number of bees are quiet. Crawling continues for several hours, accompanied by a gradual weakening and loss of definiteness in the movements. In from one to four hours the crawlers become too weak to stand or crawl normally and move with a staggering motion. They frequently fall and eventually become unable to right themselves. During the crawling period bees sometimes lose the use of one or more legs. The affected legs are dragged or used ineffectively in crawling. Weakness increases until the infected bees are capable of only feeble movements of the legs, mouth parts, and abdominal segments.[3]

Bees that are naturally infected or are artificially infected within the colony show the same symptoms of disease as are shown by bees kept in cages. As soon as attacked by the fungus, field bees fly or crawl from the hive and usually die at a considerable distance from the entrance. During weather favorable for flight probably not more than 5 or 10 per cent of the infected bees die within or directly in front of the hive. Most of these die at night and are young bees

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[3] These observations are comparable to those made by Turesson (26) on the toxicity of molds to the honeybee and the cause of bee paralysis. As a result of his experiments Turesson concluded that paralysis among bees is caused by the eating of fungous spores and mycelium. He claims to have found very little difference in the toxicity of the different species that he tried and believes that toxic substances are present in most fungi. He speaks of observing the germination of spores and development of mycelium within the alimentary canal, but apparently did not suspect any of the organisms capable of parasitizing bees. The writer's conclusions differ from the conclusions of Turesson on this point.

that were infected before their first flight. These young bees avoid the light and consequently remain in the hive until the disease is well advanced. In artificially infected colonies the first crawlers appear in from one to three or four days, depending largely upon the virulence and rate of growth of the fungus, and the quantity of inoculum used. Bees were seen to leave the hive and fly heavily. Some fly for only a short distance, then fall to the ground as if too weak to maintain flight. Several such short flights may be made by diseased bees while sufficient strength remains. When too weak to fly, they crawl and frequently attempt to take wing from the tops of grasses or stems, but fall to the ground only to try again. Death usually occurs within a few hours after they have come to the stage where they are no longer able to fly. When not too weak, the crawlers taken from the shade into bright sunlight or a warm room may recover sufficient strength to take wing again. This apparent recovery lasts for only a short time, and death occurs as soon as if the bees had remained exposed to the lower temperatures. Cowan (*9, p. 189*) attributes the loss of flight in bees infected with *Mucor mucedo* to pressure exerted by developing mycelium and the consequent inability to distend the air sacs. In the case of Aspergillus infection, however, a general weakening as a result of disease seems to account for their inability to fly.

No important changes in appearance occur at death. The body of the bee retains its normal color and shape. The abdomen may or may not be distended, since this depends upon the quantity of food consumed by the diseased bee just previous to death. The body appendages retain their normal position in relation to the body, which may lie in any position. A slight increase in the hardness of the abdomen may have occurred at death, though this hardening has been immediately preceded by a softening of the infected tissues. Soon after death, if the original infection was heavy and the saprophytic development of putrefactive bacteria is not too rapid, the abdomen becomes noticeably harder than in normal bees or in bees dead of disturbances not of a fungous nature. The muscles of the thorax, on the other hand, usually become considerably softer soon after death, as can be noted by crushing them between the fingers or under a cover glass. This character is of little diagnostic value, however, as the same symptom is present in bees dying of other causes. The increase in firmness of the abdomen at this time is, therefore, the only distinctive external symptom of fungous infection. After death the firmness remains or increases, and the tissues of the abdomen may become quite hard. The thoracic muscles, after first softening, also increase in rigidity, and then upon drying become of chalklike consistency.

When the abdomens of these dead bees are dissected for microscopic examination the digestive tract and surrounding tissues are found to be tough in texture and are less easily crushed than in the case of healthy bees or of bees dead of other diseases. While the ventriculus of the healthy bee spreads evenly when pressed under a cover glass, the ventriculus of a bee dead of mycosis offers considerable resistance to pressure. Teasing it apart with needles is often necessary to prepare mounts that will transmit sufficient light for satisfactory examination.

When dead bees infected by aspergillus are kept in a moisture-saturated atmosphere at ordinary temperatures the fungous growth continues after death and attacks all of the softer tissues of the abdomen, thorax, and even the head. Tufts of mycelium and conidiophores develop at the body openings. Conidiophores also grow through the thinner parts of the exoskeleton, at the articulation of the body appendages, and at the junction of the abdominal segments. (Pls. 3, A and B; 4, A; and 5, A.) When conidiophores do not develop upon the surface of the dead bees they may do so inside the exoskeleton of the abdomen or thorax. (Pls. 4, B, and 5, B.) Conidiophores do not develop in a very dry atmosphere, whereas an excess of water aids the growth of bacteria and thus prevents the development of conidiophores.

After inoculation and before symptoms of mycosis have appeared the spores present within the alimentary canal increase in size as a result of imbibition of water, except within the honey stomach, where the contents are usually highly concentrated. When several times their original size the spores germinate, and an extensive mycelium develops. (Pl. 6.) Spores that germinate within the ventriculus may limit their growth for a time to the food contents, but the wall is soon penetrated by the developing mycelium. A similar process of development may occur within the pharnyx, proventriculus, small intestine, or rectum, and less frequently within the honey stomach. The Malpighian tubules usually remain free from attack until after the death of the bee. The trachea likewise remain free from attack, even in advanced stages of decay after death. Spores appear to be unable to germinate within the trachea, owing to dryness resulting from aeration. In addition, these organs are protected from perforation by the tough layers of the interior surface. The small quantity of available food-supplying material within the tracheal walls likewise limits fungous growth. All of the softer tissues of the three divisions of the body may be attacked by the fungous mycelium. The thoracic muscles are a favorable medium for the development of pathogenic species of Aspergillus.

The infection of adult bees with pathogenic fungi results normally only from the germination of spores within the alimentary canal. Spores failed to cause infection when placed on various parts of the exterior body surface of adult bees, and germ tubes from spores cultured in small quantities of nutrient media did not penetrate the tissues when placed on the soft covering of the neck or in the folds of the abdominal segments. Spores spread over the openings of the spiracles likewise failed to cause infection.

Symptoms of Mycosis in the Brood

In brood, as with adult bees, Aspergillus infection shows a number of more or less arbitrary stages, depending upon the degree of development of the infecting fungus. The changes in symptoms may occur with considerable rapidity within a period of a few days owing to the rapid growth of mycelium. Both larvae and pupae are attacked by any of several common species, but after the feeding state, and especially after the cells are capped, the chances of infection are considerably reduced. In these experiments the larvae were attacked

in greater number than were the pupae. The symptoms of disease in the larvae and pupae are quite similar; consequently no attempt is made to differentiate between them.

The symptoms manifested by infected brood before death are not readily noticeable. There may be a slight increase or alteration in movement in larvae that become infected before the quiescent period or in pupae that are infected just previous to the time for emerging. Ordinarily this passes unobserved, since the extent of the movement of which brood is capable, within the cells of the brood comb, is very limited. During their active period diseased larvae may turn to one side or the other or turn completely over, although this is not a usual occurrence. Both larvae and pupae usually die while in their normal position in the cells.

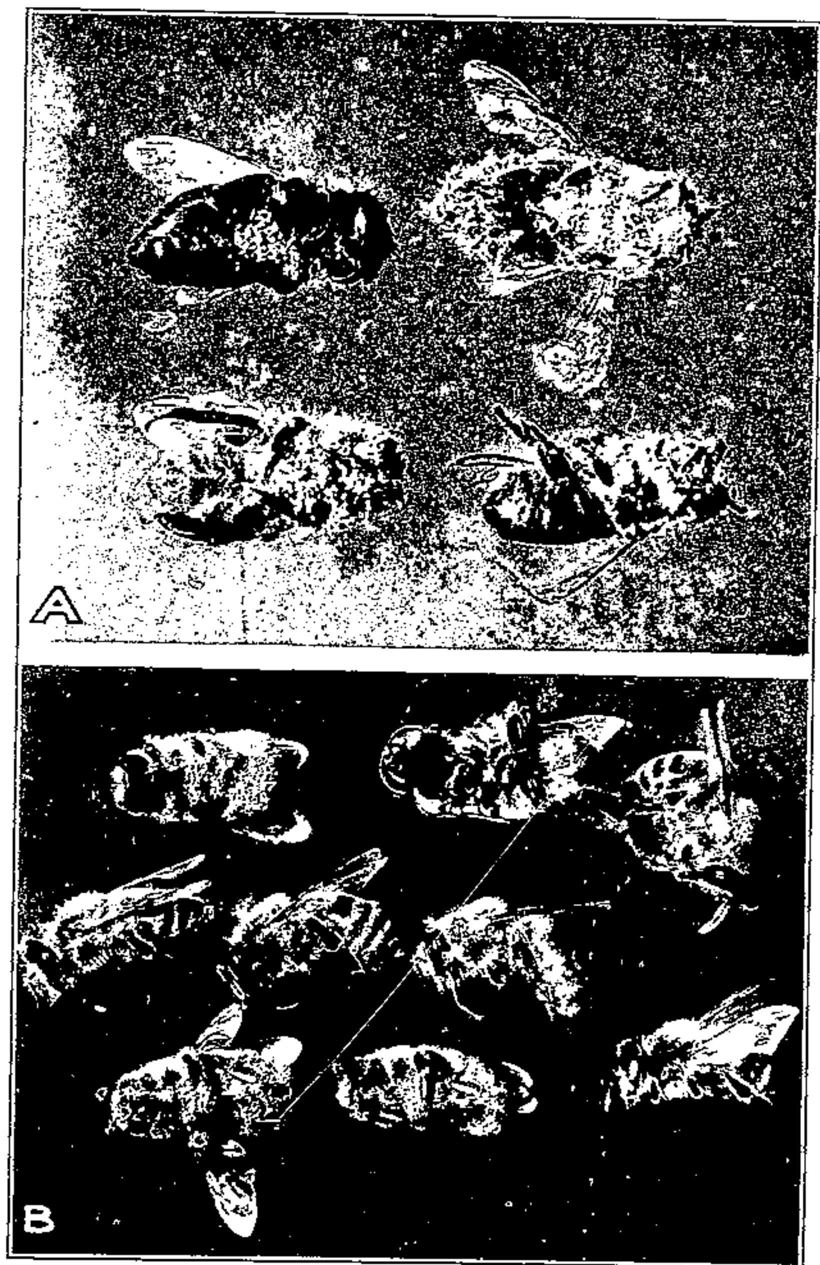
It is difficult to determine when death occurs, especially during the quiescent period. There are no changes in the outward appearance of infected brood for a short time before and after death. Larvae or pupae, at an age when they are normally capable of movement, may be assumed to be dead when all movement has ceased. The fact can be determined by examination under a reading glass which magnifies four diameters or more, or under a binocular microscope. The skin of living healthy larvae normally presents a moist and glistening appearance. Movements of the mouth parts and breathing movements are readily noticeable. With death all movements stop, and the skin becomes dry and dull after a few hours.

After death, before mycelium appears outside the skin, changes in appearance and texture develop. Firmness increases as a result of the interweaving of mycelium in and about the digestive organs and tissues. The color in this stage changes from the glistening white of the healthy larva to a dull, dry, creamy white. Drying, as indicated by wrinkling and shrinking, has already begun. The anterior end of the larva, which usually shows the first indications of drying, may be sharply curved ventrally, bringing the anterior and posterior ends closer together.

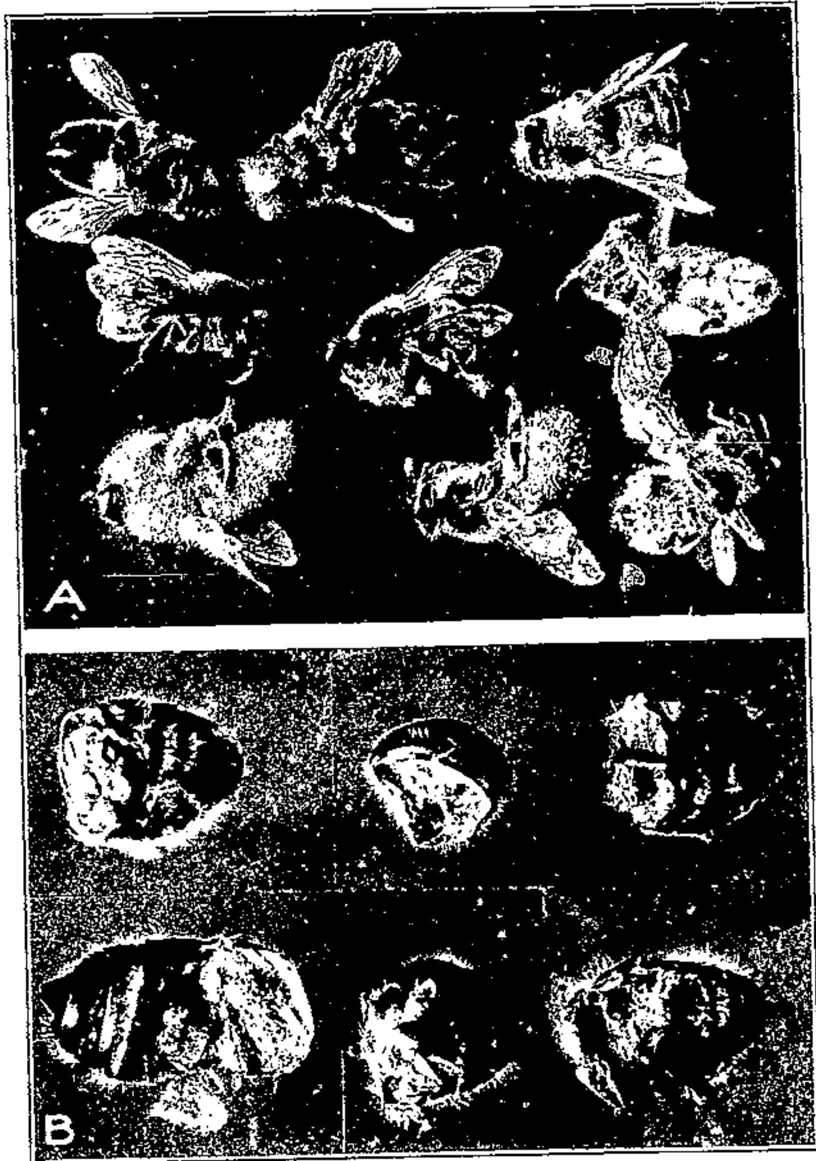
Soon after color changes appear the mycelium breaks through the skin. It appears first in a circle of radiating hyphae near the anterior extremity and gives to the dead larva the appearance of possessing a collar. Development of mycelium over the surface forms a false skin composed of closely woven hyphae. (Pl. 1, C.) The segmental markings are retained in this false skin; consequently close observation is necessary to distinguish between this and the true skin. The false skin of mycelium may be removed without injuring the true skin if care is used.

The development of conidiophores upon the surface of the larva begins at about the time of the maturation of the false skin. They form most abundantly at the anterior and less abundantly at the posterior extremities where the mycelium is in direct contact with the air. (Pl. 1, C.) When the conidiospores mature, the white color of the false skin is replaced, wherever the dead larvae are not in direct contact with the cell walls, by the color of the spores of the infecting organism. (Pl. 1, C.)

After the spores are mature changes take place much more slowly. Aging of the spores is accompanied by darkening and a loss of bril-

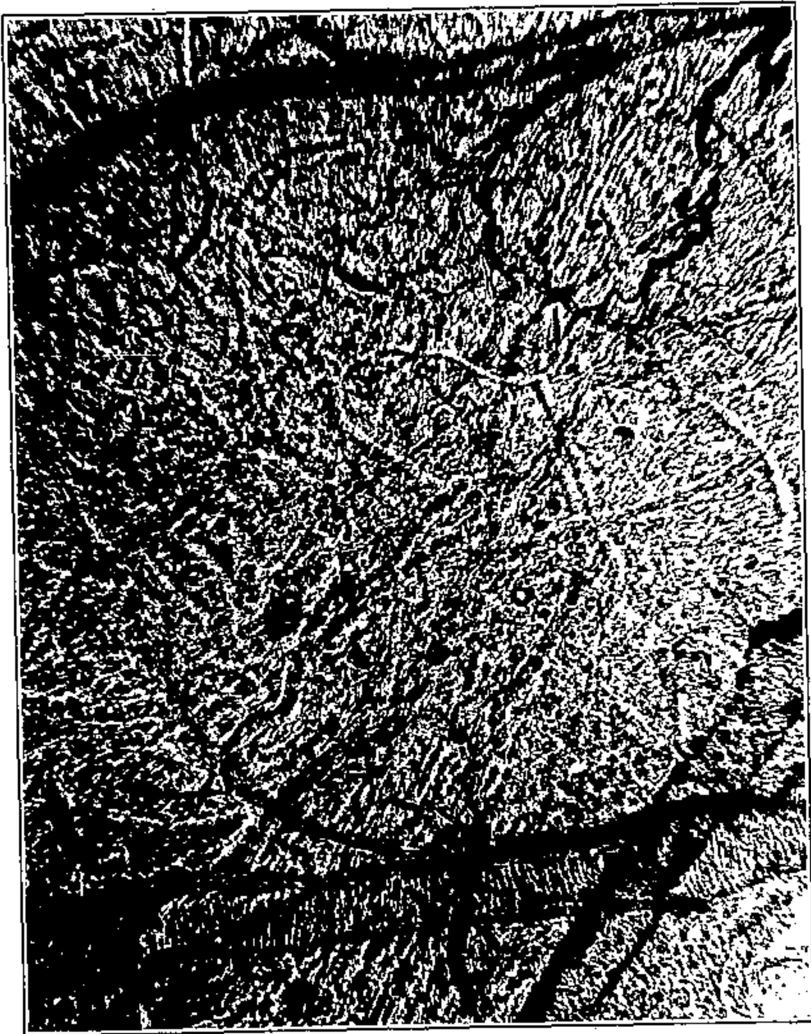


A.—Bees dead of Aspergillus mycosis caused by natural infection with *Aspergillus ochraceus*.
B.—Worker bees dead of Aspergillus mycosis caused by natural infection with *A. flavus*.



A.—Bees showing mixed infection with *Aspergillus flavus* and *A. fumigatus*.
B.—Dissected abdomens of bees dead of Aspergillus mycosis produced experimentally, the penetration of the exoskeleton by the conidiophores having been prevented by keeping the bodies in a dry atmosphere.

A. A queen pupa mummified by *Aspergillis flavus*.
B. Decapitated bee (naturally infected) showing conidiophores of *A. flavus* that matured within the thorax.



Photomicrograph of tissues of the abdomen of a bee infected with *Aspergillus fumigatus*, × 1,200.

liancy, and continued drying of the body results in the formation of a mummy of considerable hardness.

Infection of brood from fungous spores within the digestive organs occurs in a manner quite similar to the infection of adult bees (p. 19). Infection of larvae is not limited in its origin strictly to the digestive organs, although this is the usual channel of infection. Spores of two species of Aspergillus, *A. flavus* and *A. fumigatus*, placed on the skin of larvae produced local infection at the point of contact. Germ tubes penetrated the skin and attacked the subcutaneous tissues in about 15 per cent of the cases in some experiments. This mode of infection occurred in larvae in combs, and in larvae kept on honey or sugar solution in watch crystals. Soon after the skin is penetrated and the mycelium has become well established in the tissues tufts of aerial hyphae and conidiphores appear at the point of infection. (Pl. 1, D.)

Although these experiments would seem to indicate that direct penetration by germ tubes from spores germinating on the surface of larvae is possible, it probably rarely occurs. Even in the experiments where masses of spores were placed on the surface of the larvae, germ tubes generally failed to penetrate the skin.

Results of Inoculation of the Blood of Bees with Spores of Aspergillus

In the experiments with caged bees a few wounded ones were observed that appeared to be attacked in the wound by the fungus. Bees that were inoculated in the tissues or blood were killed more quickly than when spores of pathogenic fungi were taken into the alimentary canal. All of the species of Aspergillus that were found capable of attacking bees through the alimentary canal proved to be pathogenic when introduced into the tissues or blood. Other species that did not attack bees when taken into the digestive organs were found to be capable of attacking bees when inoculations were made by wounding.

This method of infection could seldom occur in nature on account of the constant protection afforded by the body covering.

Study of the Physical and Chemical Action of the Fungus on the Infected Bees

The germination of spores of filamentous fungi within the alimentary canal of bees and the growth of mycelium here and in the tissues result in disease and death. It has been stated by Vincens (*27*) that "the parasite seems to act by mechanical obstruction of the digestive passages or by paralysis of the muscles of the intestine."

As a result of the study of freshly prepared mounts, the writer believes that the blocking of the digestive tract is of minor consequence. When the first symptoms appear the quantity of mycelium present within the alimentary canal of infected bees is in most cases so small that it is difficult to understand how it can affect the movements of food. Death usually occurs before a sufficient quantity of mycelium has developed to cause congestion within the digestive tract. Frequently, when feeble movements of the appendages or abdominal segments are the only signs of life, only a few scattered hyphae can be found within the contents of the alimentary canal.

It appears that one must look for other causes responsible for disease and death in the bees infected by fungi.

To determine these causes of death, a histological and chemical study was made of infected tissues from diseased and dead bees. The chemical action of the fungus was determined by observing the infected tissues, and by testing the action of certain metabolic products produced by the fungus, when applied to the tissues in the absence of the fungus.

It was determined by examination of sections of the ventriculus that hyphae penetrate the tissues soon after the spores germinate. The wall is usually attacked first and is soon permeated with mycelium. Other tissues of the abdomen and thorax are also attacked by the fungus.

That a mechanical effect is produced in the tissues by the advancing mycelium is shown by the forcing apart of the muscle fibers when the hyphae grow among them. Later the mycelium becomes densely interwoven among all of the softer tissues, and the mechanical effect of this is probably sufficient to cause death.

In view of the rapid softening of the tissues and the appearance of weakness, with the consequent crawling condition, among infected bees, it appears that enzymatic and toxic substances contribute materially to the symptoms. The tissues of the bee are attacked by the fungous enzymes in advance of the growing mycelium, but this digestion is not completed until the mycelium is well developed. The resultant softening of the muscle tissues of the thorax is followed by a loss of the brownish tinge characteristic of healthy tissues and the appearance of a dull white color. A sarcolemma is absent about the wing-muscle fibers of the honeybee, and no support other than the sarcoplasm is known to exist. The semifluid sarcoplasm surrounding the sarcostyles is softened, permitting the muscle fibers to separate readily. This probably accounts for the softening and collapse of muscle tissue and the ready separation of the threads of muscle fibers under pressure.

Softening of the sarcoplasm surrounding the muscle fibers is followed by the death of the sarcoplasm of the sarcotyles and the disappearance of their membranous walls. In advanced stages of digestion, i. e., after death, the sarcostyles break up into segments, frequently separating at the telophragmata. After complete digestion only formless granules remain.

The muscles of the thorax are most easily studied, but the wall of the ventriculus may be used successfully since it is attacked soon after infection occurs. The digestion of sarcoplasm of the tissues of the bee is probably largely proteolytic in nature, for protein is the chief constituent of muscle tissue. It appeared, however, from some comparative measurements made by the writer that pathogenicity of the organisms studied is in no way related to the quantitative production of protease. It is possible that the sarcoplasm is killed by fungous toxins before enzymatic action begins.

The observations described above on infected tissues indicate that the changes which occur after the death of the tissues are apparently due to enzymatic action. If this is true, muscle tissue should be similarly affected in fungus-free culture media or in solutions of extracted enzymes.

In contrast to the findings of Eduard Buchner (*4*), in his work on the extraction of zymase from yeast, the enzymes of several species of Aspergillus and Mucor were found to exist in much greater quantities in the medium in which cultures have grown than within the mycelium. Evidence in support of the view that the effect of pathogenic fungi on bees is chiefly enzymatic and toxic was obtained from the following experiments:

Enzymes from the liquid medium in which pure cultures of *Aspergillus flavus*, *A. effusus*, and *A. fumigatus* had matured were extracted by precipitation with alcohol. After thorough drying the precipitate was dissolved in distilled water. The digestive tract and muscle tissues of bees that had been recently killed, entire bees, and each of the three divisions of the body were submerged in this solution and kept at 37° C. while the action was observed. Tissues directly exposed to the action of enzymes were soon discolored and in from 5 to 10 hours offered no noticeable resistance to pressure. Tissues of thoraces, abdomens, and heads of bees with the exoskeleton of each of these body divisions attached were affected similarly but somewhat more slowly. The chitinous exoskeleton appeared to be unaffected after 24 hours, whereas no evidence of enzymatic action on the internal tissues of entire bees was noticeable after 30 hours. It appears that the exoskeleton is not extensively attacked by any of the enzymes produced by these fungi, although conidiophores and mycelium penetrate the body wall of the bee when excessive moisture is present. Ordinarily the only channel of infection is through the alimentary canal.

The experiments described above were repeated with the filtered medium from cultures, with sterile medium that had not been used for culturing, and with distilled water. With the medium from cultures the action was similar but somewhat slower than with extracted enzymes. With sterile, freshly prepared medium and with water as checks, no enzymatic action upon tissues was noticeable after 48 hours. The medium, which was of a high sugar content (25 per cent), appeared to act as a preservative, since bacterial decay of the tissue occurred later than in distilled water.

Demonstration of a Toxic Substance Produced by Aspergillus flavus A050

It is a familiar fact that many of the diseases of animals and plants are due to microorganisms which elaborate toxic substances of some form or other. The exact nature of these substances, which are at times partially responsible for the symptoms of the various diseases, is not completely known. The inability of chemists to determine their constitution is probably due to the impurity, complexity, and unstable nature of the molecule. It is possible by various procedures to extract from cultures of pathogenic organisms substances that are more or less toxic. When toxins are properly administered in the absence of the pathogenic organisms they may produce the symptoms of the disease. Severe poisoning may also be produced by toxic products of nonpathogenic organisms. The potency of these toxins, which may be many times as effective as strychnine, is well known. Toxic substances are not uncommon among the

higher plants, and among the mushrooms there are numerous species whose toxins have caused the deaths of great numbers of mushroom eaters. Although numerous species of the Hyphomycetes are active parasites of other fungi, higher plants, and animals, very little has been done to demonstrate the nature of the toxic substances elaborated by these organisms.

In the progress of some plant diseases caused by fungi the cells of the host are killed or altered far in advance of the developing mycelium of the parasite. This is particularly true among the rusts where zones of dead cells may surround the infected spot. The death of the cells of the host not in direct contact with the fungus can probably be attributed to toxic products of the parasite.

A few reports of toxic substances produced by filamentous fungi are on record. In 1906 Paladino-Blandini (*20, p. 608*) prepared an alcoholic precipitate from the mycelium of *Rhizopus nigricans* which was toxic to rabbits when injected intravenously. In 1915 Blakeslee and Gortner (*3*) published a more complete study of this toxin and its action upon rabbits. In earlier experiments by these two investigators (*11*), results with rabbits were negative when the fungus was administered by feeding in large doses. (Such an alcoholic extract would be a protein, and its nontoxic nature when fed can be explained by its digestion to nonpoisonous compounds before absorption.) In 1896 Gosio (*12*), working with a species of Penicillium, found that the culture medium gave phenol reactions. When injected into rabbits and rats, phenol poisoning resulted. In the study of maize deterioration, Alsberg and Black (*1, p. 13, 43*) isolated a characteristic phenolic substance from *Penicillium puberulum* and *P. stoloniferum*. This substance could only be isolated from the medium. Turesson (*26*) as a result of his work on the toxicity of fungi concludes that it is of wide occurrence in fungi.

To determine whether or not such a toxic substance injurious to living bees is produced by these fungi experiments were begun with two pathogenic organisms, *Aspergillus flavus* Ao5c (Thom collection) and *A. fumigatus*, isolated from an infected worker bee.

Direct injection into the blood of bees was considered impracticable; consequently feeding experiments were adapted as a test for the toxicant. Food prepared by the addition of honey to the medium in which these Aspergilli had been grown and to juices pressed from mycelium was fed to caged bees. When the unheated medium or fungous juice from cultures was used it was necessary to filter it, using the best grade of filter paper, to eliminated spores of the pathogenic organisms. This was partially accomplished only with *Aspergillus flavus*. *A. fumigatus* was not used after the first attempt on account of the small size of the spores. Although the death rate among the bees fed with medium in which cultures had matured averaged higher than among the checks which were fed freshly prepared medium, the method was discarded because the death rate among the checks, due to salts, was higher than normal. When bees were fed with the fluids pressed from mycelium, or with the wash water from the mycelium, the death rate remained about normal. It appeared, therefore, that if a toxic substance is produced, it must be sought in the medium rather than in the mycelium of the fungus.

EXTRACTION OF THE TOXIN FROM THE MEDIUM

It appeared, as a result of the experiments discussed above, that the toxic substance must be extracted in order to avoid error from other factors that affected the death rate. Since nothing was known of the nature or constitution of the toxic substance, it was necessary to determine the best method for extracting it.

Alcohol extracts

Extraction of proteins with alcohol was tried first. The precipitates obtained from the medium in which fungi had been cultured and from the juices pressed from the mycelium after grinding it with sand were dissolved in water and fed with dilute honey to bees in cages. The bees took this food readily, but the death rate remained normal throughout two trials. It is not proved by these experiments that a poisonous protein is not produced, since such a poison may be digested to nonpoisonous compounds before absorption, as with higher animals (*11, p. 357*). On the other hand, absorption by the honeybee of some of the simpler sugars appears to be a question of only from one to a few minutes.

Ether extract

The medium from 10-day-old flask cultures of *Aspergillus flavus* Ao5c and the fungous juices pressed from the mycelium after it had been washed and ground with sand were shaken five times with small quantities of ether. After each shaking the ether was separated from the medium with a separatory funnel. The ether was then washed with a large volume of distilled water, separated, and evaporated to dryness in evaporating dishes. Brown amorphous residues were obtained in larger quantities from the medium than from the fungous juices. Each of the residues was dissolved in 5 cubic centimeters of distilled water and added to about 5 cubic centimeters of clover honey. This was given as food to caged worker bees. The bees that received the extract from the fungous juices lived normally; but some of the bees receiving the extract from the culture medium were noticeably affected after 4 hours, and after 10 hours all that had been fed with this extract were dead. The experiment with the culture medium extract was repeated with four lots of bees, the same food being used for each succeeding lot after all of the bees in the previous lot had died. A constant decrease in potency was noticed with each successive trial. With the second trial all of the bees were dead at the end of 18 hours; with the third, after 30 hours; with the fourth, after 2 days; and with the fifth, after 7 days. (Table 1 and fig. 1.)

This experiment was repeated several times with similar results except that the earlier deaths were not obtained again in succeeding experiments. Extracts from old cultures that had been kept at room temperature and from young cultures prepared at about the time spores were forming appeared to contain only small quantities of the toxic substance. The greatest accumulation of toxin within the medium seems to be present at about the time the cultures are

mature, as indicated by the appearance of a deep yellow-green color in most of the conidia. The disappearance of the toxin from cultures and the rapid loss of potency after extraction indicate that it is of a transient nature. This is shown in Table 2 and Figure 2. When freshly prepared ether extract was fed to bees the entire lot died in

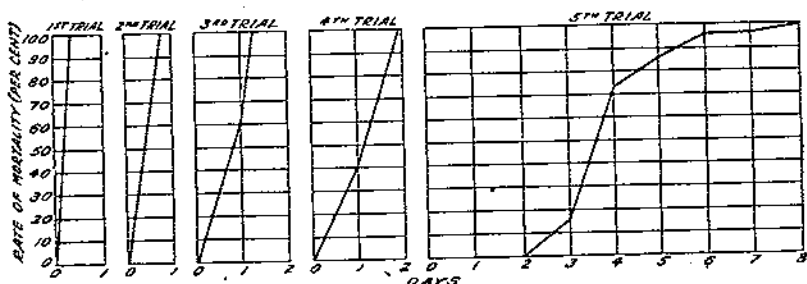


FIGURE 1.—Rate of mortality of honeybees at daily intervals after the commencement of feeding with honey containing ether extract of the nutrient medium in which *Aspergillus flavus* had been cultured. The food was transferred to a new cage of bees as soon as the bees of the previous trial were dead

five days. Fifteen days later the same food was given to a second lot of bees. This trial showed a death rate that was nearly normal for caged bees.

TABLE 1.—*Length of life of bees in cages when ether extract of the culture medium of Aspergillus flavus Ao5c was mixed with their food*

Age of extract when fed to new lot of bees	Number of deaths on days stated								Total deaths
	1	2	3	4	5	6	7	8	
Freshly prepared	[1] 24								24
24 hours	[1] 26								26
2 days	15	10							25
4 days	10	16							26
8 days	0	0	5	16	4	3	0	1	29

[1] Although this table indicates only that the bees of the first and second tests were dead after 24 hours, the actual time was 10 hours in the case of the first trial and 18 hours in the second.

TABLE 2.—*Length of life of bees in cages when ether extract of the medium in which Aspergillus flavus Ao5c had been cultured was mixed with their food*

Age of extract when supplied to a new cage of bees	Number of deaths on days stated																					Total deaths
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Freshly prepared	7	4	4	0	1																	25
16 days	0	0	0	0	0	1	1	2	0	1	1	2	2	1	3	3	3	2	2	1	1	26

Chloroform extract

To find whether poisonous organic bases or other poisonous substances soluble in chloroform were present, the liquid nutrient medium (Leonian's formula) was thoroughly drained from half a dozen flask cultures of the mature organism, *Aspergillus flavus* Ao5c.

The fungous fluids were pressed from the mycelium after it had been washed and ground with sand. Both the medium and the fungous fluids were each shaken five times with small volumes of chloroform and separated with a separatory funnel after each operation. After washing in an evaporating dish the chloroform was evaporated to dryness, and the residue from each solution was taken up with about 5 cubic centimeters of distilled water and given to worker bees with an equal volume of honey. In each case the entire quantity of food was consumed without evidence of poisoning. Repetitions of this experiment at different stages in the development of the fungus gave entirely negative results. It would seem therefore that bases capable of poisoning bees when administered with food are not produced by *A. flavus* Ao5c.

Chemical Nature of the Toxic Substance

In most cases chemists have been unable to determine the constitution of toxic substances elaborated by microorganisms; therefore only

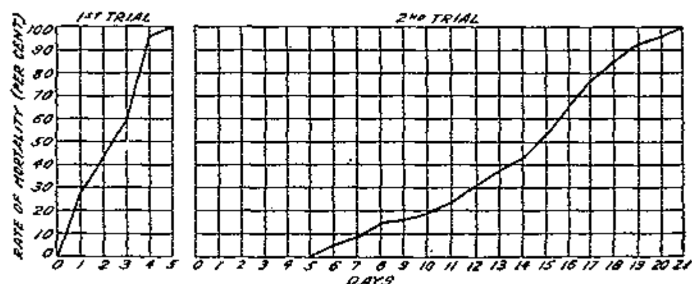


FIGURE 2.—Rate of mortality of honeybees at daily intervals after the commencement of feeding with honey containing ether extract of the nutrient medium in which *Aspergillus flavus* had been cultured. First trial made immediately after the extract was prepared, second trial 15 days after the extract was prepared.

a few tests were made to determine the general nature of this substance. Tests for phenolic compounds were made by floating small quantities of freshly prepared ether extract on very dilute ferric chloride solution. The absence of color reaction in all cases indicated the absence of such compounds in the extract. When the extract from flask cultures in 5 cubic centimeters of water was mixed with 5 cubic centimeters of honey, the resulting solution showed a hydrogen-ion concentration of about pH 4.6 by the colorimetric method of Clark and Lubs (*7*) and Clark (*6*), whereas equal volumes of water and the same honey showed a concentration of about pH 5.

Something of the nature of the toxic substance is shown by the following experiments: Freshly prepared extract was divided into three equal parts. One part was fed directly to bees in cages. The other two parts were made neutral, or slightly basic, by the addition of potassium hydroxide. One of these parts was immediately adjusted by hydrochloric acid to a hydrogen-ion concentration of about pH 4. 6. Honey was added to each of these two lots and they were fed to bees. The bees given the original unneutralized extract showed definite evidence of poisoning after 24 hours. The death rate was considerably higher than normal, and all of the bees had died

before the end of the fifth day. Bees fed upon honey with the ether extract, which had been made neutral with potassium hydroxide, showed a normal death rate for 15 days, when all of the food was consumed. Bees given the solution that had been readjusted to about pH 4.6 after neutralization died at about the same rate as bees fed on the ether extract that had not been neutralized. (Table 3 and Fig. 3.)

TABLE 3.—*Length of life of bees in cages when ether extract of the culture medium of Aspergillus flavus Ao5c was mixed with their food*

pH value of the food containing fungous extract	Number of deaths on days stated															Total deaths
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Freshly prepared; about pH 4.6	0	4	6	6	3	6	----	----	----	----	----	----	----	----	----	25
Made basic with potassium hydroxide	0	3	1	0	0	0	0	0	1	0	0	2	1	3	(1)	11
Changed from basic to pH 4.6 with hydrochloric acid	0	5	6	6	2	3	0	3	----	----	----	----	----	----	----	25

[1] The total number of bees used in this experiment was also 25. The food containing the fungous extract was consumed by the bees after 14 days, when records of the daily death rate were discontinued.

If, as is indicated by the foregoing experiment, the toxic substance is of basic nature, it should be dissolved out with chloroform if the medium is first made basic. Potassium hydroxide was added to the medium from 12-day-old cultures until it gave a distinct basic reaction. Chloroform was then shaken with the medium, separated, and evaporated to dryness, as was done with ether. A flocculent precipitate, which formed upon the first addition of chloroform, was separated, drained, and dissolved in a small quantity of water. By the addition of honey to this precipitate a food for bees was prepared. A food was prepared also with the residue left upon evaporation of the chloroform. No poisoning resulted from either food. After six days each preparation was adjusted to a pH value of about 4.6 by the addition of sulphuric acid and fed to recently caged bees. The death rate in both cages remained normal. The toxic substance, if present at first, appeared to have been entirely destroyed in the basic condition.

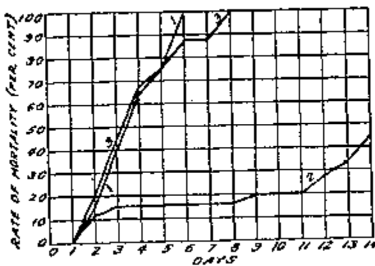


FIGURE 3.—Rate of mortality of honeybees at daily intervals after the commencement of feeding with honey containing ether extract of the nutrient medium in which *Aspergillus flavus* had been cultured: 1, With freshly prepared extract; 2, with extract made slightly basic with potassium hydroxide; 3, with extract adjusted to a concentration of about pH 4.6 after it had been made slightly basic

That it was nearly all removed from the medium was shown by adjusting the medium to its original pH value and extracting with ether immediately after the extraction with chloroform was complete. When this ether extract was fed to bees the death rate was about normal.

In order to determine whether the toxic substance could be recovered by the adjustment of the pH value immediately after

extraction with chloroform, an extraction was made from a new set of cultures. As soon as the chloroform had evaporated the residue was taken up with water, mixed with honey, and adjusted to about pH 4.6. The precipitate that formed with the first addition of chloroform was treated in the same way. The death rate was increased when either the residue left after evaporation of the chloroform or the precipitate was fed to caged bees. The death rate from the residue was much higher than that from the precipitate, which shows that there is a greater amount of toxin in the chloroform. (Table 4 and fig. 4.) It may be that the presence of the toxin with the precipitate can be accounted for by adsorption.

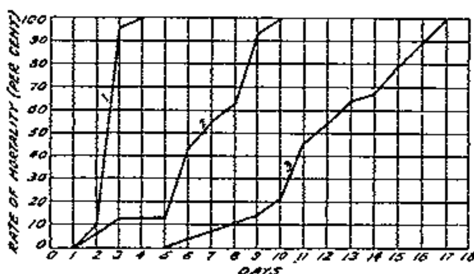


FIGURE 4.—Rate of mortality of honeybees at daily intervals after the commencement of feeding with extract of the medium in which *Aspergillus flavus* had been cultured: 1, With chloroform extract from medium made slightly basic with potassium hydroxide before extracting. The food containing the extract was then adjusted to a concentration of about pH 4.6 with sulphuric acid just before it was given to the bees. 2, With the precipitate obtained upon the addition of chloroform to the basic medium. The food containing the extract was adjusted to a concentration of about pH 4.6 before it was given to the bees. 3, With ether extract of the medium adjusted to an acid reaction after extractions had been made with chloroform

TABLE 4.—*Length of life of bees in cages when the extract from the culture medium of Aspergillus flavus A05c was mixed with their food*

Method of preparation and pH value of the extract	Number of deaths on days stated																	Total deaths
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Chloroform extract adjusted to pH 4.6	0	3	26	1	---	---	---	---	---	---	---	---	---	---	---	---	---	30
Precipitate obtained with chloroform (adjusted to pH 4.6)	0	1	1	0	0	5	2	1	5	1	---	---	---	---	---	---	---	16
Ether extract from medium after extracting with chloroform and adjusting to original pH value	0	0	0	0	0	1	1	([1])	2	2	7	([1])	5	1	([1])	([1])	0	28

[1] Records of the death rate were not made on this day.

As checks upon the experiments, extractions were made with ether and with chloroform from medium that had not been used for culturing. These extracts were added to diluted honey and fed to bees. All of this food was consumed, but none of the bees appeared to be poisoned since the death rate was normal. (Table 5 and fig. 5.)

TABLE 5.—*Daily death rate of bees in cages when given honey for food*

Investigator	Cage No.	Number of deaths on days stated																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Phillips	1	20	0	0	4	2	12	2	3	0	8	4	6	11	10	9	3	1	---	---
Do	2	1	0	4	28	12	0	2	5	4	0	6	6	8	2	3	2	2	---	---
Burnside	3	0	0	0	0	1	3	6	1	7	3	3	1	3	3	1	5	7	3	2
Do	4	0	0	0	0	0	0	[1] 1	1	2	([2])	5	3	2	0	1	1	([2])	2	4

[1] Three other check lots of 25 each showed that the first deaths of normally fed bees occurred on the sixth or seventh day.
[2] Records of the number of deaths were not made on these days.

TABLE 5.—*Daily death rate of bees in cages when given honey for food*—Contd.

Investigator	Cage No.	Number of deaths on days stated																		Total deaths
		20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
Phillips	1																			101
Do	2																			100
Burnside	3	2	1	1																53
Do	4	2	5	5	(1)	(1)	6	0	5	11	10	4	2	1	5	1	3	0	1	83

[1] Records of the number of deaths were not made on these days.

The graphs of Figures 1 to 4 and Tables 1 to 4 show the rate of mortality obtained under varying conditions when fungous extracts mixed with dilute honey were fed to bees in cages.

The normal death rate for caged bees when given honey as food is shown in Table 5 and in the graphs of Figure 5. Graphs 1 and 2 of this figure were drawn from data obtained by Phillips (*19, p. 395–399*) on the death rate of bees in cages when given honey for

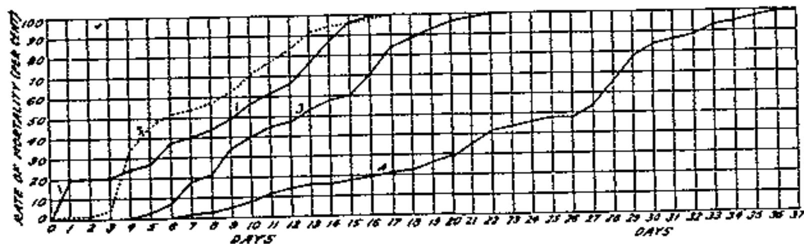


FIGURE 5.—Rate of mortality of lots of honeybees in cages when fed with honey. This graph represents normal death rates for bees kept in cages

food. Graphs 3 and 4 were drawn from data obtained by the writer while determining the normal death rate of bees in cages, as checks upon other experiments. These cages were kept in the laboratory on the opposite side of the room from the source of light. The bees were supplied with honey diluted with an equal volume of water. The temperature of the laboratory during the time that the experiments were in progress ranged between 23° and 27° C.

In view of the difference in death rate between the experiments of Phillips and those of the writer, when bees in cages were given honey for food, one is led to believe that there were important differences in the conditions of the experiments. Either the bees used by Phillips were not entirely healthy or the conditions under which his experiments were conducted were less favorable for caged bees.

RESULTS OF EXPERIMENTS WITH THE PENICILLIA

While studying the fungi associated with honeybees the writer (*5, p. 64*) found that species of Penicillium caused the mummification of bees more often than did any of the other forms. Bees mummified by Penicillia resemble closely those killed by Aspergilli. Attempts were made to infect healthy bees with cultures of Penicillium isolated from other bees. Adult bees in cages and in colonies were unaffected when spores of these organisms were fed to them. Adult bees and brood in colonies exposed to the cultures of Penicillium on

brood combs were also unaffected. Bees were attacked and killed, however, when the spores of an unidentified species of Penicillium, isolated from mycelium growing in a wound of a living bee, were placed in wounds made with a needle. The infection was at first localized in the wound, but soon spread to other parts of the body. Nothing further could be done with this species though the following members of this ubiquitous group of fungi were tested for pathogenicity: *Penicillium corylophilum* Dierckx, *P. cyclopium* Westling, *P. palitans* Westling, *P. expansum* Link sensu Thom, *P. commune* Thom, and *P. brevicaule* Saccardo sensu Thom.

Although the spores of the above species of Penicillium did not germinate within the digestive tract of living bees, they remained viable and often germinated soon after the death of the bee, causing mummification of the tissues. This probably accounts for the similarity of conditions observed in bees mummified by Penicillia with those conditions observed in bees killed by Aspergilli.

RESULTS OF EXPERIMENTS WITH THE MUCORS

Adult worker bees were inoculated with species of the genus Mucor which had been isolated from bees mummified by these organisms. They were unaffected in most of the experiments, though an occasional bee was attacked by one species, *M. hiemalis* Wehmer, when bees were caged on cultures, or when spores of this organism were mixed with dilute honey and fed to them.

Only an occasional bee was attacked in colony experiments in which most of the honey was removed from the hives and replaced with honey diluted with three volumes of a water suspension of spores of *M. hiemalis*. The brood in such cases was unaffected. The other species isolated from bees and tested for pathogenicity on bees in cages were *M. racemosus* Fresenius, *Rhizopus nigricans* Ehrenberg sensu Lendner, and *Syncephalastrum racemosum* F. Cohn. None of the bees were attacked by these.

SYMPTOMS PRODUCED BY OTHER FILAMENTOUS FUNGI

A considerable number of fungi belonging to genera other than those already discussed have been isolated from bees and tested for pathogenicity by feeding spores to bees, or by caging bees on the cultures. Among the fungi tested were *Fusarium negundo* Hubert and other unidentified species of Fusarium, unidentified species of Sporotrichum, *Cladosporium herbarum* Pers, *Hormodendrum atrum* Bonord, and *Myceliophthora inflata* Burnside. Only negative results were obtained in the experiments with these fungi. An unidentified species of Isaria, isolated from a sowbug, and *Metarrhizium anisopliae* (Metsch.) Sorokin attacked less than 10 per cent of the inoculated bees.

Other fungi have been isolated that were found vegetating within the digestive tract or tissues of sick bees. Although the observations made indicated that some of these were pathogenic to the bee, inoculation experiments were limited to those forms that appear on bees with considerable frequency.

RESULTS OF EXPERIMENTS WITH THE YEASTS

In the experiments with yeasts, ordinary baker's yeast, certain identified yeasts isolated from bees, and several unidentified species, were used. The specific identifications were determined by comparison of the morphological and cultural characteristics of the yeast with those given for these species by Guilliermond (*13*). Introductions through the alimentary canal were made by mixing cultures of yeast with the food, by caging bees on the cultures, and by wetting them with water suspensions of yeasts. Inoculations were made directly into the blood of bees through wounds made with a sterile needle. The foods with which the yeasts were mixed included dilute honey, 10 to 20 per cent sugar solution, and Laurent's medium for yeasts to which about 20 per cent of sugar or honey was added.

A dysenteric condition appeared among caged bees that were heavily inoculated by feeding on *Saccharomyces ellipsoideus* Hansen, *S. cerevisiae* Hansen, and on two cultures of *Mycoderma cerevisiae* Desm. and Hansen that differed in cultural characteristics. Uninoculated bees kept under conditions as nearly identical as could be obtained were not similarly affected. No effects were noticeable from feeding on *Saccharomyces apiculatus* Klöcker and one other unidentified, slow-growing yeast. The death rate among inoculated bees averaged higher than among the checks for the entire series of experiments, although the difference was so small that its significance is as yet uncertain.

When bees were heavily inoculated by feeding on *S. cerevisiae* and *S. ellipsoideus*, taken from cultures on potato slants, a condition resembling intoxication developed. Gas, which gave positive tests for CO_2, was formed within the ventriculus of the intoxicated bees. Recovery from this condition, however, occurred within three or four hours, and the death rate among the bees remained normal when the food containing yeast was replaced with pure honey.

Caged bees were unaffected when small quantities of yeast were mixed with their food, and bees in colonies were apparently unaffected when the only food available consisted of dilute honey or sugar sirup with which half a dozen yeast cultures were mixed.

Limited growth was observed to occur within the ventriculus of infected bees when their food consisted of dilute honey sirup or Laurent's medium. Growth never progressed farther, however, than the formation of one or two buds by less than 20 per cent of the cells. In about 3 or 4 days after bees were given the cultures most of the yeast had passed from the ventriculus into the rectum. Yeast cells within the contents of the rectum were found to be viable up to 10 days after being eaten.

When the inoculation with yeasts was made in the blood by punctures in the thorax, infection and death resulted in from 50 to 100 per cent of the individuals. With *Saccharomyces apiculatus* death resulted in about 50 per cent of the cases. Weakening and crawling of some of the inoculated bees was first noticed after 2 days, and death usually occurred in less than 24 hours after the first symptoms were apparent. Most of the inoculated bees that were infected died in from 2½ to 6 days after they were inoculated. A few died of disease after 8 days, although as a rule those that survived for 8 days after inoculation had not been infected.

Other yeasts isolated from bees, including *S. ellipsoideus*, *S. cerevisiae*, *Mycoderma cerevisiae* I, and *M. cerevisiae* II, three unidentified yeasts, as well as brewer's yeast, gave results similar to those with *S. apiculatus*.

The highest death rate was obtained with *S. ellipsoideus* and *S. cerevisiae*. With both of these organisms the first symptoms of disease appeared in from 24 to 36 hours at normal room temperature, although at 30° C. this time was shortened. The highest death rate occurred during the second to fourth day after inoculation, and a few bees died between the fifth and sixth days. With these two organisms from 75 to 100 per cent of the bees inoculated usually died from infection.

With *Mycoderma cerevisiae* I, *M. cerevisiae* II, and the three unidentified yeasts from bees, the death rate from infection was slightly higher than with *Saccharomyces apiculatus*. Infected bees rarely lived longer than seven days after the inoculations were made. From 50 to 70 per cent of the inoculated bees died as a result of infection.

Microscopical examinations were made of the thoracic muscles and blood of inoculated bees at intervals after inoculation. Yeast cells multiplied rapidly within the blood and at the time of death were present in such large numbers that the blood appeared milky. Although the organism was ultimately carried to all of the body divisions by the blood, the most abundant development seemed to occur on the muscle fibers of the thorax. Yeast cells seemed to multiply there while the blood flowed over them without altering their position.

In a few cases the infection appeared more or less localized in the region surrounding the point of inoculation. With *S. apiculatus*, small spindle-shaped to linear pockets filled with yeast cells were observed in considerable numbers between the fibers of the wing muscles and apparently between the sarcostyles near the surface of the muscle fibers. There were usually from 5 to 20 yeast cells in each of these pockets at the time death occurred.

Symptoms were readily discernible upon dissection of the bees after death. The blood was found to have lost its pale-brownish color and had become milky. At the time of death, the wing muscles were slightly less rigid than in normal bees. Later they became mummified and brittle. The drying wing muscles shrink away from the exoskeleton except where they are attached. One of the most distinctive symptoms of yeast infection determinable without the aid of a microscope is the presence of a chalk-white coating on the surface of the muscles after they have dried. The presence of numerous yeast cells within the blood and on the muscle fibers is readily established with the aid of a microscope. No external changes were observed by which bees dead of infection with yeasts could be distinguished.

DISCUSSION

These investigations have dealt with the fungi for which pathogenicity has been established and those that are closely associated with bees but usually do not parasitize them. All of these ordinarily reach their typical development as saprophytes under a wide variety

of conditions. Some of them occur regularly on brood combs under proper conditions for their growth, whereas others are rarely found there.

The specific descriptions have not been included, since they are obtainable in standard works on mycology.

The Aspergilli are widely distributed, and the various species develop under widely different conditions. They are most commonly met with as saprophytes growing upon rotting fruits and other food products in which there is a high concentration of sugars. During dearths of nectar, spores of Aspergilli may be gathered by bees with juices of fruit or other juices which contain sugar. They are often found on the brood combs under certain conditions of moisture which exist in weak colonies, or in hives in which colonies have died out during winter or spring. Wet extracting combs when stored in damp rooms may also be attacked by a number of the species. Many of the species of Aspergillus, however, reach their typical development under dry conditions. Grain in storage, dried fish, foods, and herbarium specimens are frequently attacked by these. Some forms are typical inhabitants of the soil. Still others are capable of becoming active animal parasites, attacking insects, birds, and mammals, including man.

Of the several hundred isolations of Aspergilli from bees affected by mycosis, more than half were yellow-green spored forms of the *Aspergillus-flavus-oryzae* group. The greater number of these in turn were of the *A. flavus* series with comparatively long conidiophores. This may probably be accounted for by their virulent pathogenic nature, their abundant spore production, and their widespread saprophytic growth on a variety of natural substances including extracting and brood combs. All of the organisms belonging to this group isolated from bees from the chief beekeeping sections of the United States and from Canada and Europe have been found to be pathogenic when tested. Tests with a considerable number of races, strains, or species of the group, obtained elsewhere than from bees, seem to indicate that there is considerable difference in the degree of virulence. Intermediate strains, all known as *A. flavus-oryzae*, have been isolated from bees on but few occasions. These organisms, when tested, attacked both bees and brood. Intermediate forms from other sources have also given positive results, but the death rate among bees was generally lower than with *A. flavus*. Other strains failed to attack bees under similar conditions.

A. oryzae was not isolated from bees, but at least one strain, *A. oryzae* (113 L), obtained from the Thom and Church collection, attacked a few bees in cages and an occasional larva when spores were shaken over a brood comb containing uncapped brood. It is only among the *A. flavus* series of this group, however, that pathogenic organisms are found that are of importance as causing bee diseases.

The deep-green strains, which produce acid most abundantly are the ones that attack bees most readily. On the other hand, the thickness of the spore wall, which in *A. flavus* is about twice as thick as in *A. oryzae*, may be a determing factor in protecting the spores from the digestive fluids of the bee until germination can take place.

Organisms belonging to the *Aspergillus fumigatus* group occur on adult bees (pl. 4, A) and less frequently on the brood. Tests for pathogenicity with *A. fumigatus* isolated from bees obtained from widely scattered sections of the United States have all given positive results. Cultures obtained from the collection of Thom and Church and from a prominent drug manufacturer of Detroit have been found capable of parasitizing bees. Negative results have never been obtained with *A. fumigatus*, but brood-comb cultures placed in colonies caused less damage than was caused by *A. flavus*. In cage experiments the mortality from the fungus has usually been 100 per cent.

Aspergillus nidulans occurs less frequently on bees than does *A. fumigatus*. Isolations have been made from adult bees from time to time and once from brood. Inoculation experiments with *A. nidulans* from bees and from other sources seem to indicate that a number of strains are pathogenic to bees. The death rate among bees inoculated with *A. nidulans* is lower than with *A. flavus* or *A. fumigatus*, and the bees frequently escape infection.

Aspergillus glaucus has been isolated from mummified larvae and from adult bees. In experiments with caged bees only a small percentage were infected, and cultures obtained from the Thom and Church collection failed to attack any of the bees. Brood was not attacked when spores were scattered over larvae from two to four days old.

The black-spored Aspergillus, *Aspergillus niger*, has rarely been found on bees, although isolations have been made from adult bees and from mummified larvae, and a few worker bees were attacked in inoculation experiments. Attempts to cause infection of brood by *A. niger* were not made. Such an extreme case as that described by Morgenthaler (*18*), in which the comb cells were filled with a loose, dark-brown or black powder and larvae shriveled, but with swollen edges, lay covered with this dust, has never been observed in these experiments. The brood combs of tightly closed hives from which the bees were removed have been observed to be completely overgrown with *A. niger*. No noticeable infection occurred, however, when these combs were given to strong colonies even though brood rearing was begun in them within a few days.

In one instance an organism identified as *Aspergillus ochraceus* appeared to have been the cause of heavy losses in a Connecticut apiary. Examination of samples sent to the writer showed that more than 75 per cent of the diseased bees were attacked by this organism. (Pl. 3, A.) A second lot from the same apiary yielded about 60 per cent of infected bees. Inoculation experiments with young cultures were unsuccessful, as neither bees nor brood were attacked. When year-old cultures that had thoroughly dried were used, all of the inoculated bees were attacked and killed.

It was found that young but apparently mature spores of this Aspergillus were not only unable to germinate within the alimentary canal but were killed after a short time. No growth occurred after the bees were killed, and the spores failed to germinate when placed on nutrient agar. Gorging with young spores was ineffective in stimulating an attack. During the culture of this organism the spores were observed to be at first thin walled with a gelatinlike

coating. Later on the spore walls became thicker and roughened while changes of a chemical nature result in hardening of the gelatinous covering. This additional protection in old spores from the digestive fluids of the bee may explain the ability of old spores to attack bees and the complete lack of this ability in young spores. After germination, the digestive fluids of bees appear to have no injurious effects upon the young mycelium.

Two other cultures of *A. ochraceus*, isolated from bees but differing in morphological and cultural characteristics from this form, failed to parasitize bees or brood regardless of the age of the spores.

A few species of Aspergillus, among them *A. versicolor*, *A. terreus*, *A. candidus*, *A. sydowi*, *A. sulphureus*, and *A. clavatus*, isolated from beehives or bees, failed to infect bees in cages. Colonies of bees, therefore, were not tested with these organisms. The mycelium of these species was superficial on bees, and the characteristic mummification, common in mycosis, was absent. If these species ever attack bees, it would appear that special conditions are necessary.

The Penicillia appear to have only a purely saprophytic relation to bees. The resemblance of bees mummified by any of a number of species of Penicillium to those dead of mycosis may be explained by the rapid saprophytic growth within the bee immediately after death.

The frequency with which the Penicillia occur on bees may be accounted for, at least in part, by their ubiquitous nature and by the fact that the spores are not quickly killed by the intestinal fluids. Viable spores in large numbers are usually present on the brood combs and equipment within the hive. When Penicillium spores gain entrance to the alimentary canal before death, they may grow saprophytically within the bee after death.

The only damage to bees for which the Penicillia are responsible is caused by their saprophytic growth within the hive. Since the conditions of moisture and temperature within strong, well-managed colonies rarely permit the growth of fungi on the combs, damage is generally limited to poorly ventilated hives, weak colonies, and combs in unoccupied hives.

Mucors have been found to occur commonly on bees, but the experimental evidence thus far obtained indicates that ordinarily they are not pathogenic. Sporangiospores may swell considerably or even bud once or twice in a yeastlike manner within the ventriculus without seriously injuring the bee. It was found by cultures from the ventriculus and rectum of inoculated bees that sporangiospores of most of the Mucors investigated were soon killed by the intestinal fluids of adult bees. Inoculated bees that later died of starvation were not attacked by the Mucor after death.

It appears, however, that, under certain conditions which as yet have been only partially determined, bees are parasitized by one or more species of Mucor. In the inoculation experiments with *Mucor hiemalis* an occasional bee died of infection. This species has often been isolated from mycelium found vegetating in sick bees and from dead bees.

The results of investigations with this Mucor will be given in another paper.

Inoculations with other filamentous fungi that commonly occur on bees have always given negative results; consequently the writer is

of the opinion that they rarely attack healthy bees. Occasionally fungi differing from those found to be pathogenic by experimental inoculation have been isolated from mycelium found vegetating within the digestive tract or tissues of sick bees. This would seem to indicate that the fungi observed could parasitize the bees. However, since no one species other than those already discussed was isolated more than two or three times, their pathogenic relation to bees was not extensively investigated.

The yeasts have been observed to occur with considerable constancy within the alimentary canal of bees. The number of cells present was usually small, and they were often limited to the contents of the rectum, but a considerable number of cases have been observed in which much of the contents of the honey stomach or of the ventriculus consisted of yeast cells. In most of these cases the cells were evenly distributed within the contents, and, although buds in various stages of development were present, it could not be ascertained whether growth had occurred within the bee. At one time yeast, in more or less rounded hard masses of the size of wheat grains, was found in the honey stomachs of more than 10 per cent of a lot of sick bees collected in November, 1924, at Ann Arbor, Mich. Three distinct yeasts, one of which was identified as *Saccharomyces ellipsoideus*, were isolated from the masses of yeast cells. It was thought possible that these yeasts had developed within the honey stomachs of the bees and were responsible for their abnormal condition.

A series of feedings with these yeasts and others, including *Saccharomyces apiculatus*, isolated from bees failed to justify such an assumption. None of the yeasts multiplied extensively within the alimentary canal, and the bees were not seriously affected by the presence within the digestive tract of large quantities of yeast. Masses of yeast cells, such as were found within the honey stomachs, did not form there within these inoculated bees. Such masses did form, however, but without apparent harm, within the ventriculus of bees that were heavily inoculated. In this case the yeast masses were doubtless formed as a result of the sifting out of the cells from the food contents of the ventriculus. Such a process could also occur in nature when bees feed upon partly fermented honey.

Yeasts have been found, often in large numbers, in the tissues of bees several days after their death. Among these, *Saccharomyces apiculatus* and *S. ellipsoideus* were recognized. A small oval yeast that was not identified was found in the thoracic muscles of about 50 per cent of a sample of nearly a hundred dead bees sent to the bee-culture laboratory from South Carolina. The gross symptoms of these infected bees resembled closely those of bees inoculated with yeasts by being punctured with a needle that had been dipped into a suspension of yeast cells.

Following these observations on dead bees, it seemed desirable to observe living bees to determine if yeasts are capable in nature of parasitizing healthy bees by gaining entrance into their blood. Although many apparently affected bees were examined, yeasts were never found vegetating in the blood.

Although it could not be determined whether or not the yeasts found in the muscle tissues and blood of dead bees had been the

cause of death, all of the methods of infecting other than the puncture method gave negative results.

In summing up the evidence from the experimental inoculations with yeasts and the observations on bees, it appears that ordinarily yeasts do not infect the blood or tissues of healthy, uninjured bees, although they are capable of becoming actively pathogenic when they gain entrance to the blood or tissues.

It has been shown that certain species of fungi are pathogenic to honeybees of all ages. Some of these attack adult bees in nature with considerable frequency and reach a degree of economic importance when conditions favor the production of large numbers of spores. Others attack bees so rarely under normal conditions that their importance as causative agents of disease is negligible, whereas still others that have never been found on bees in nature were found to attack bees when inoculated experimentally.

The greater number of fungus species that cause diseases of adult bees also attack the brood. This was found to be the case with most of the species or strains of Aspergillus used in these investigations. When inoculated experimentally with a pathogenic Aspergillus, brood is attacked and killed more quickly than are adult bees, though the loss of brood resulting from Aspergillus mycosis is much less than that of adult bees.

The frequency with which bees are attacked by fungi in nature appears to depend chiefly upon the virulence of the pathogenic species and upon their dispersion. Conditions that favor abundant growth of pathogenic fungi in nature are conducive to the spread of fungous diseases. The fact that brood is rarely attacked can probably be explained by the small probability that larval food will contain a sufficient number of viable spores to cause infection.

The pathogenicity of a fungus appears to be determined by the ability of its spores and mycelium to resist the action of the intestinal fluids within the digestive tract of bees.

Mycosis of bees reaches its greatest significance with adult workers. Throughout the active season an appreciable number of these are killed by pathogenic fungi, principally by the yellow-green spored Aspergilli. In one case about 40 per cent of a lot of bees that showed symptoms similar to mycosis yielded pathogenic species of Aspergillus. Usually, however, only about 5 per cent of the suspected bees were infected.

It is difficult to estimate the importance of mycosis among bees because adult bees generally die away from the hive, and, when the percentage remains small, larvae may be carried out soon after they become infected. The death rate from mycosis during the winter varies with conditions within the hive. In strong colonies under good wintering conditions it is negligible; in weak colonies, when pathogenic fungi have grown over the combs, mycosis may become an important factor in the dwindling and death of the colony.

In view of the fact indicated by this study, that fungous diseases of bees are not likely to assume the importance of either of the foul broods, methods of control have not been extensively studied.

Complete control would be difficult to attain, since worker bees may be infected while gathering sweet juices from fruits, food products, and many other natural and prepared substances, upon which

pathogenic fungi grow. Infection from this source, however, is not likely to become epidemic.

Infection from molded combs, equipment, and dead bees can be considerably reduced by care about the apiary. During the winter ample provisions should be made for the escape from the hives of metabolic water vapor. The bottom board should be cleaned of molded bees as early in the spring as weather conditions are suitable for manipulating the bees without danger of chilling them. Brood combs and extracting combs, when not in use, should be stored in dry rooms to prevent pathogenic fungi from growing on them.

In order to prevent the possible occurrence of disease, all badly molded combs and equipment used inside of the hive should be dipped for a few minutes in a 20 per cent solution of formalin in water or exposed to formaldehyde gas in an air-tight chamber for two or three days. Molded hive bodies, bottom boards, and covers which can not be dipped readily should be washed with the same solution.

SUMMARY

This investigation of the fungous pests of bees shows that parasitic fungous species occur on bees and brood, that they may cause quite virulent diseases, that some of these forms reported as pathogenic in Europe also occur in North America, and that there are additional pathogens not heretofore reported. Certain other mycological and biochemical data have been obtained and presented; and the effects of the invading fungi on the bees, their development, and the disturbances caused within the body of the bees and larvae, have been worked out in considerable detail.

The fungi shown to be pathogenic include species of Aspergillus and Mucor, and the Saccharomycetes. Several species of Aspergillus and one of Mucor are most important.

Members of the *Aspergillus flavus* series were shown to attack bees more frequently than other forms of Aspergillus, but *A. fumigatus* is also virulently pathogenic. In addition, *A. nidulans*, *A. niger*, *A. glaucus*, and *A. ochraceus* attack bees in nature.

Bees are attacked when spores of pathogenic fungi are taken into the alimentary canal. They can be artificially inoculated by mixing spores of the fungi with their food or by causing them to come in contact with mature cultures. Infection also results when spores are placed in the blood of bees by puncturing the exoskeleton.

The spores germinate within the food contents of the alimentary canal. The hyphae penetrate the wall, and under favorable conditions all of the softer tissues are attacked by the mycelium. The spores of nonpathogenic fungi do not germinate within the alimentary canal of healthy bees, but with some species they remain viable and germinate after the death of the bee.

Germ tubes were found not to penetrate the exoskeleton of healthy adult bees. They may be induced to penetrate the skin of larvae, but this rarely, if ever, occurs in nature.

Before death the gross symptoms of mycosis are not distinct from those of other disturbances in adult bees. At the time of death, or soon thereafter, positive diagnosis can be made microscopically from

the presence of mycelium within the tissues. Diagnosis may also be made from the post-mortem changes.

The action of pathogenic fungi upon the tissues of bees is both physical and chemical in nature. Tissues are penetrated by the developing mycelium and are digested by the fungous enzymes. One pathogenic fungus, *Aspergillus flavus* Ao5c, was shown to produce a transient toxic substance which is the cause of fatal poisoning in bees.

None of the species of Penicillium encountered attack healthy bees in nature. *Mucor hiemalis* attacks bees under proper conditions, but several other species of Mucor studied appear to be harmless.

Yeasts were found to be more or less constantly present in the alimentary canal of bees, although, except in extreme cases, bees are normally unaffected by their presence. A number of yeasts, however, including *Saccharomyces ellipsoideus*, *S. cerevisiae*, *S. apiculatus*, *Mycoderma cerevisiae*, and baker's yeast, are pathogenic when introduced into the blood of bees. Yeasts rarely if ever gain entrance to the blood of healthy bees in nature; consequently they appear to be of little significance pathogenically.

Filamentous fungi, differing from those investigated, have also been observed vegetating in tissues of sick bees, but inoculations have uniformly given negative results.

The observations made thus far tend to indicate that bees become infected from beekeeping equipment, dead bees, and molded combs, and from molded fruit and other substances that contain a high concentration of sugar.

Badly molded combs and equipment within the hives of infected colonies should be disinfected by being dipped or washed in a 20 per cent solution of formalin in water or by being exposed to formaldehyde gas.

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