The University of Chicago
FOUNDED BY JOHN D. ROCKEFELLER

THE IMMUNOLOGICAL REACTIONS OF OIDIOMYCOSIS (BLASTOMYCOSIS) IN THE GUINEA-PIG

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE OGDEN GRADUATE SCHOOL
OF SCIENCE IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

(DEPARTMENT OF PATHOLOGY AND BACTERIOLOGY)

BY

BENJAMIN FRANKLIN DAVIS

Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. 8, No. 2, March 1911, pp. 190–240
Chicago, 1911

THE IMMUNOLOGICAL REACTIONS OF OIDIOMYCOSIS (BLASTOMYCOSIS) IN THE GUINEA-PIG.*

BENJAMIN FRANKLIN DAVIS.

(From the Department of Pathology and Bacteriology, the University of Chicago, Chicago, Ill.)

The object of this work was to examine the mode of resistance of guinea-pigs to oidiomycetic infection; to determine whether it might be possible to increase this resistance by immunization, and, if so, to investigate the factors upon which this increase in resisting power depended.

LITERATURE.

For a full discussion of the relation of oidioid and blastomycetoid organisms to human and animal diseases and for literature as to their relative pathogenicity for various animals, the reader is referred to the work of Ricketts,[11] Brown,[25] Hektoen,[26] Montgomery and Ormsby,[27] and Spiethoff.[19] The papers considered here are those which have dealt somewhat specifically with the question of the mode of resistance of animals to infection with mold fungi, and with the demonstration of antibodies in the infected animals.

Metchnikoff[1] (1884) demonstrated that phagocytosis is the chief means of defense of the daphnia against infection with a mold fungus christened by him "Monospora bicuspidata." This was followed by a number of articles by Ribbert[2] (1887) who was inclined to believe that mold fungi (Schimmelpilze) and pathogenic schizomycetes injected into rabbits are ingested by leukocytes within which they undergo intracellular digestion. Charrin and Ostrowsky[4] (1896) observed that immunized animals were but moderately resistant to reinfection with *Oidium albicans*. Roger[5] (1896) obtained considerable resistance to *Oidium albicans* by vaccination. Rabbits given repeated intravenous injections of sublethal doses became able to resist infection by double the lethal dose. In normal serum the organisms grew quite readily; in immune serum they were first agglutinated, then became hyaline, the capsule disintegrated, and later attempts to obtain cultures showed that the oidia were dead. Schattenfroh[6] (1896), using a non-pathogenic yeast, found that the sera of animals were not bactericidal but that peritoneal exudates were markedly so. He concluded that the germicidal power of peritoneal exudates depends on phagocytosis. Gilkinet[7] (1897) believed from the results of his experiments, (1) that beer yeasts (*Saccharomyces cerevisiae*) introduced into rabbits either intravenously or subcutaneously produce

* Received for publication, June 25, 1910.

neither local nor general symptoms; (2) that such yeasts do not multiply in living tissues; (3) that they are destroyed within a very short time, and cannot be discovered in the bodies of the animals by any method; (4) that this destruction is brought about by the substances in the plasma and is not a function of the body temperature, of chemical reaction, or of the absence of nutritive substances, but is a specific, unknown property of the organic fluids; (5) that this destructive property is not dependent upon the formed elements of the blood, but is exercised in the same degree by all the body liquids; and, finally, (6) that this property is itself destroyed by heating at 55° C. He found that yeast mixed with ox serum or rabbit serum and kept at 36° C. for from two to four days lost its power of growth on suitable media, but that if such mixtures were kept at room temperature for corresponding periods, the yeasts were unharmed. He placed porous tubes containing blastomycetes in the peritoneal cavities of three rabbits. The first rabbit was examined after four days; the yeast cells were not much changed, and were not in contact with leukocytes; in the second rabbit, killed after nine days, and in the third rabbit, killed after twelve days, there were but few normal yeast forms and apparently all were dead, since cultures were negative. Leukocytes were not in contact with the organism. The author produced edema of the rabbit's leg by tight bandaging; such edematous fluid. free from leukocytes, killed yeasts in three days at 37° C. Jona[8] (1897) gave rabbits intraperitoneal, intravenous, and subcutaneous injections of a non-pathogenic yeast, *Saccharomyces apiculatus*. He concluded that in all cases the organisms were destroyed within a few hours through the influence of the body fluids. Organisms injected into the peritoneal cavity, or subcutaneously, did not make their way into the blood. Obici[9] (1898) found that after repeated injections of the toxin (filtered broth culture) and small quantities of the spores, an immunity was established in rabbits, against *Aspergillus fumigatus*. This immunity, however, was incomplete, and vanished in a short time. He did not believe, with Ribbert, that phagocytosis was of such paramount importance in the defense of the animal body against this infection, as he saw evidences of degeneration in organisms in the absence of phagocytic cells. Skchiwan[10] (1899) experimented on guinea-pigs and rabbits with *Saccharomyces tumefaciens* of Curtis. He concluded that the body fluids are not bactericidal since the organisms protected from the leukocytes by celloidin sacs grew well on artificial media after being in the peritoneal cavity of the animal for four days. On the other hand, yeasts injected directly into the peritoneal cavity were phagocyted by certain cells and digested. The phagocyted yeasts lost their staining power in from two to four days, as well as their power to grow when planted on suitable media. The leukocytes formed rosette-like masses about the organisms. At first polymorphonuclear leukocytes were found in such masses; later, the groups consisted entirely of macrophages. Malvoz[13] (1901) experimented with six strains of yeast, some pathogenic and some non-pathogenic. The pathogenic cultures he obtained from Sanfelice, Plimmer, Curtis, and one he isolated himself from an epithelioma, calling it *Blastomyces E* (BE) as a convenient laboratory name. His non-pathogenic cultures consisted of a strain of *Saccharomyces ellipsoideus* and a powerfully fermentative organism which he called the yeast of Huy. Rabbits were given two weekly injections of the organism of Huy, BE, and Sanfelice over a period of months, and the agglutinative properties of the sera were then tested. The specific serum agglutinated the organism of Huy in a dilution of 1:50; organism BE, 1:90; organism Sanfelice, 1:5. The specific serum for the organism of Huy agglutinated *S. ellipsoideus* in the same titre as the organism of Huy itself; more

weakly the organism of Plimmer and organism BE; and the organism of Curtis not at all. The specific serum for organism BE agglutinated organism of Huy very slightly. The organisms of Huy, Sanfelice, and organism BE grew well in normal and immune serum, and grew on artificial media when transplanted after 1 to 24 hours. The author concluded that in this case either bactericidal antibodies were absent or the organisms were protected by their capsule. He suggests that the Bordet-Gengou reaction might be of service in deciding the presence or absence of such antibodies.

Ricketts[11] (1901) says: "Professor Hektoen has found that the undiluted serum of a dog which had received successive inoculations of the organism from Case I (cutaneous oidiomycosis) causes gradual clumping of the organism diffused in bouillon. Several hours elapse before the fullest extent of clumping possible is reached. Organisms from other cases show only a slight degree of clumping with the same serum. All organisms grew well in the serum and the production of mycelium was especially noticeable. Normal dog's serum caused no clumping of any of the organisms; abundant mycelium is produced in all cases.

"The organism from the case reported by Hyde, Hektoen and Bevan was repeatedly inoculated into the abdominal cavity of a rabbit. It was found that the animal's serum would cause fairly distinct agglutination of the organism inoculated."

Sanfelice[3] (1896) discovered that heating for 30″ at 60° C. destroyed the virulence of his *Saccharomyces neoformans* for guinea-pigs, but did not destroy its power to grow on artificial media. He endeavored to immunize guinea-pigs against this organism (1) by the injection of organisms whose virulence had been destroyed by heating to 60° C. for 30″; (2) by repeated injections of filtered broth cultures of various ages; and (3) by the injection of the serum of guinea-pigs and of dogs which had received repeated injections of either the heated organism or the filtered culture, or had recovered from the infection induced by a dose of virulent organisms. He was wholly unsuccessful.

In 1902 Sanfelice[15] was able to immunize dogs, cats, and rabbits to *Saccharomyces neoformans*, Plimmer's yeast, and to a non-pathogenic yeast isolated from the air, so that the animals could withstand, without symptoms, an intravenous injection of quantities of the first two organisms which caused the death of normal, control animals. He had no difficulty in demonstrating the presence of a specific amboceptor in the serum of such immune animals by the fixation test. He was unable to demonstrate the antibody in actively infected animals which subsequently died of the disease. Unfortunately he never took the trouble to control his fixation experiments by testing the effects of his antigen—emulsion of yeast—or of his antibodies—inactivated immune and normal sera—upon the hemolytic system which he used—serum of rabbits immune to fowl corpuscles—a fact which detracts considerably from the value of his results. He says that Malvoz[12] demonstrated antiblastomycetic amboceptors by the fixation reaction in 1901. Sanfelice believed that the immune sera caused a change in the yeast cells exposed to their action such that the organisms assumed an appearance identical with the so-called Russell's fuchsin bodies of malignant tumors.

Wlaeff[16] (1902) immunized geese and donkeys to a yeastlike organism which he isolated from the ascitic fluid of a patient whose disease had been diagnosed as inoperable abdominal cancer with ascites. A piece of the growth, removed at an exploratory operation, was diagnosed by Cornil as a typical cylindrical-celled carcinoma. The sera of the immunized animals agglutinated and dissolved the specific organisms. Wlaeff claimed that, following repeated injections of such sera, the ascitic fluid of this

cancer patient likewise developed the property of agglutinating and dissolving the blastomycetes and that the cancer improved as the result of such treatment.

Fabozzi[18] (1905) concluded from his experiments that *Saccharomyces neoformans* never causes the development of tumors, and is finally destroyed by phagocytes.

Christensen and Hektoen[21] (1906) say: "The character of the lesions of blastomycosis, the accumulation of leukocytes, the formation of giant cells, and the phagocytosis of blastomycetes—indicates that this is an infection in which phagocytosis is an important means of defense and healing. Certain preliminary test-tube experiments showed that phagocytosis of blastomycetes is favored by the presence of normal serum, and the idea arose that it might be possible to stimulate the greater formation, in cases of blastomycosis, of the body that promotes phagocytosis (opsonin), as well as the other antibodies, by the injection of blastomycetic substances in a readily absorbable form. It was thought that the resistant character of the microorganisms, coupled with their inclosure in cellular exudate and granulation tissue, possibly prevents the absorption in proper quantities of the substances necessary to call forth strong immunizing reactions. Hence, in order to hasten, if possible, the reactions that favor healing, we injected in each of our cases a sterile blastomycetic vaccine prepared by Dr. H. T. Ricketts of the corresponding organism. Unfortunately the patients left the hospital at a time when no conclusions of value could be drawn as to the results of the vaccine."

Marco del Pont[23] (1907), using *Endomyces albicans*, grew broth cultures with increasing doses, first, of normal, and later, of immunized rabbit's serum. The development of a mycelium indicated that the medium was unfavorable. After the 60th transfer the yeast grew normally in undiluted immune serum. It retained this faculty. The immunity was specific; yeast habituated to rabbit serum did not grow in the serum of the dog, goat, or rat. The immunized fungus had lost its virulence. The author thought that the immunity to the serum might be due to the development of a mucilaginous capsule by the organism; he also thought that a substance anti-sensibilisatrice might be produced by the microorganism.

Widal, Abrami, Joltrain, Brissaud, and Weill[29] (1910) have found that the blood serum from cases of sporotrichosis possesses the power of agglutinating the spores—but not the cell bodies—of the *Sporotrichum schenchii* in dilutions of from 1:300 to 1:800. The agglutinability of the spores varies with the age of the culture and with the kind of medium, but not with the strain of the organism (10 strains were compared). The Bordet-Gengou reaction was constant. Among 168 normal subjects and subjects suffering from other diseases, they found an occasional agglutination or fixation reaction, but both never occurred in the same individual. The two reactions, therefore, control each other. Experimentally, two dogs and four rabbits were used. In dogs it was easy to produce infection with the organisms, and the agglutination and fixation reactions were constant and marked. In rabbits, intravenous injections of large quantities failed to cause infection. The agglutinative power of the serum increased appreciably; it varied from 1:10 to 1:30 in the different rabbits before injection, and rose, following injection, as high as 1:3,000 in one case and to but 1:300 in another. The reaction of fixation, which was tested parallel with the agglutination tests, gave "very incongruous results." No further details of these results are stated.

They found that the agglutination and fixation reactions disappeared in man after the infection was conquered. The fixation reaction disappeared more rapidly

than the other. They concluded that, so long as both are found, an active lesion is indicated; the disease is not really cured.

Co-reactions.—The organisms tested which did not give co-agglutination and co-fixation reactions with immune sporothrix serum and vice versa were the trichophytons, the organisms of Erythrasma, Favus, and of animal aspergillosis. The reactions of co-fixation and co-agglutination were obtained with organisms isolated from cases of actinomycosis and thrush (*Oidium albicans*). The serum from the case of infection with *Oidium albicans* agglutinated the spores of the sporothrix 3 times as strongly as it did the oidium itself, which was agglutinated only in dilutions of 1:10 to 1:50. The co-fixation reaction was positive with the following cultures of yeast: the organism of Curtis, of Blanchard, of Plimmer, *O. luteum*, *S. granulatus*, *S. lithogenes*, and *S. caprae*, using sera from cases of sporotrichosis, actinomycosis, and thrush. The organism of actinomycosis itself was not agglutinated by the specific serum.

Rothe[28] (1909), noting the preliminary reports of Widal,[29] tested the agglutinating power of the blood serum from two cases of actinomycosis upon the spores of the sporothrix and found them positive in dilutions of 1:160 and 1:200 respectively.

MATERIALS AND TECHNIC.

The organism used was obtained in 1905 by Dr. Rosenow from a case of oidiomycosis in the Presbyterian Hospital of Chicago. It had, therefore, been growing on artificial media for three years before the present work was begun. The stock culture was grown on a 1 per cent glucose, 1 per cent acid agar, and also in nutrient broth of similar sugar and acid content. On acid-glucose-agar, at room temperature, the organism grows in the form of long hyphae which burrow peripheralward through the superficial layers of the agar. No aerial hyphae are developed excepting in those cultures which have been started in the incubator and are later placed at room temperature. The characteristics of these hyphae will be discussed later.

In acid-glucose-broth, at room temperature, the organism grows in the form of fluffy balls, the size which the latter may reach apparently being limited only by the volume of the broth, the capacity of the retaining vessels, and the number of colonies which develop. There seems to be no production of gas. In old cultures a heavy membrane forms on the surface of the broth, and the medium, though remaining clear, assumes a dark amber color. Under the low power of the microscope in a hanging-drop preparation kept at room temperature and first observed October 20, 1908, one of these "fluffy balls" appeared as a mass of mycelial threads arranged in two zones, a central mass of tangled threads and granu-

lar material too thick for light to pierce and so not discernible in detail, and a peripheral zone of radiating threads. These threads presented a rather homogenous appearance, were quite refractive to light, and possessed a slightly greenish color by transmitted light. Their central ends were lost in the central mass; their peripheral ends were bluntly rounded. Branches were few and these few were found toward the base of the radiating threads; the branches were equal in diameter to the parent stem, were of uniform thickness throughout their length, for the most part, and were given off, sometimes at right angles, but usually at an angle of about 45°. The contour of the angle was not that of a sharp corner, but rounded. No segmentation of the hyphae nor lateral conidia were observed. No distinct cell wall appeared. On October 22, two days later, the following notes were made: Hyphae have at least doubled in length; three zones can now be distinguished in the colony: (1) a central, practically opaque zone; (2) a middle zone of branched, interlacing hyphae bearing lateral conidia; most of these hyphae show marked but somewhat irregular segmentation; and (3) a peripheral zone similar to the one described on October 20. The lateral conidia arise by short stalks from alternate sides of the hyphae, one conidium to each segment. In some cases no structures are discernible within the conidia; at other times they seem to be filled with sharply defined spherules. The segments consist of oblong, clear areas separated from each other by discs, cubes, and cylinders of a homogenous, highly refractive, greenish substance very similar in appearance to the material in the young hyphae described two days before. The peripheral ends of the hyphae and those intermediate portions in which the segmentation is but slightly marked contain scattered small, spherical, highly refractive bodies which seem to be attached to the inner side of the double-contoured cell wall which has now become visible. The hyphae are reaching far out into the vaseline by which the cover glass is attached.

November 29.—No change in method of development.

In agar hanging-drop preparations kept at room temperature essentially the same appearances are obtained as in the broth. Sometimes the lateral conidia appear to be filled with sharply out-

lined, homogenous spherules. If such conidia are kept under observation for a few weeks, the spherules can be seen to gradually fuse into larger and larger masses until finally the conidial content consists of a single homogenous mass. A somewhat similar condition has been described by Bowen and Wolbach.[20] In the case described by these authors, bodies were found on agar tubes, which were filled with refractive spherules. When these bodies were placed on fresh media the spherules fused before mycelial formation began.

On acid-glucose-agar at 37° C., the organism grew exclusively in the budding form, that is, like yeast, for about fourteen months, when it rather suddenly—in the course of a month or six weeks—developed a propensity for growing in the hyphal form to which it has clung tenaciously since. While growing in the budding form the colonies which formed on the agar were soft, yellowish white, raised, and button-shaped, consisting of a flat central area surrounded by a thickened, rounded border. They were circular in outline and from 1–7 mm. in diameter. The individual cells were from 10–30 μ in diameter.

With the assumption of the hyphal form of growth, the organisms became prone to the development of aerial hyphae. This tendency was especially noticeable in cultures kept at about 34.5° C.; 36–37° C. and room temperature (about 19–20° C.) appeared to be very much less favorable to their production, excepting in cultures, as mentioned above, which had been transferred from the incubator to room temperature. To the naked eye, the hyphae—developed at 34.5° C.—appear as very delicate stalks perhaps a millimeter in height (estimated), white by transmitted light, and having a tendency to assume a silvery sheen by reflected light. In the mass, they present a dead white, somewhat fuzzy appearance, resembling a piece of high grade, heavy filter-paper. About the edges of the growth, where the hyphae are less numerous, the cultures have a "woolly" appearance. In this condition the culture is less adherent to the medium than at earlier stages—the hyphae begin to appear about 10 days to two weeks after inoculation—and may be peeled off in a single layer taking with it pieces of agar. At a few scattered points hyphae may be seen which are three or

four times the ordinary length and project above the general level at odd angles, like the poles from the top of an Indian wigwam—probably the "porcupine" appearance mentioned by Hamburger.[22]

Under the microscope the hyphae appear as slender, hollow rods, 1 to 2 μ in diameter, with a very delicate wall. They contain a very fine granular substance in addition to quite highly refractive, homogenous, greenish spherules which vary in diameter from a fraction of a micron to one micron. Sometimes the hyphae are segmented and bear lateral and terminal conidia, as well as occasional enlargements of the individual segments, in the course of the tube itself. The conidia and other swellings are from 5 to 10 μ in diameter and contain, usually, one, and occasionally 3 or 4 spherules, similar in appearance to those noted in the bodies of the hyphae, but of considerably larger size, 4 to 7 μ in diameter. In the larger conidia, the double-contoured membrane which forms their wall is very conspicuous. Some of the largest conidia are empty; their walls are nearly 1 μ in thickness and present breaks in their continuity. Free in the suspending fluid, 0.85 per cent NaCl solution, about the hyphae under examination, are numbers of spherules exactly similar in appearance to those described within conidia. Their origin from the latter seems probable. They frequently present a marked Brownian movement.

In acid-glucose-broth cultures a granular sediment forms in the course of a week or ten days, the overlying fluid remaining clear. On shaking, the tube becomes diffusely clouded but resumes its original appearance after standing a short time. Microscopically the typical budding yeast forms appear. Occasionally elongated forms suggesting hyphae are found. The same change has overtaken the broth cultures that has been mentioned as occurring in the agar cultures, that is, the organisms no longer grow in the budding form but have reverted to the growth characteristic of room temperature, namely, the mycelial form. Whether or not it will be possible to induce the organism to resume the former mode of growth at incubator temperature (37° C.) is a question which must be left for further observation.

No attempt has been made to determine the finer details of the biology and morphology of this organism, since it is believed

that enough has been said to warrant the assumption that we are dealing with an organism which undoubtedly belongs to that genus of the pathogenic mold fungi placed by Ricketts,[11] provisionally, among the oidia, and which therefore may be considered a fair sample of the parasites responsible for oidiomycosis (blastomycosis) in man.

Besides the organism itself, a so-called oidiomycetic "extract" was made use of in the following experiments. This "extract" was made according to a method which has been in use in this laboratory for a number of years, and which is as follows:

The organism, in order to obtain considerable quantities, is planted on a large covered plate or in flat-sided, wide-mouthed bottles. When a good growth has appeared, which may take from three weeks to a month, it is scraped off by means of a glass or platinum hoe and placed in a desiccator where it is allowed to dry. The dried material is weighed, then placed in a porcelain-ball mill together with an equal volume of sterile sand and about 10 c.c. of sterile 0.85 per cent salt solution and ground for a couple of hours. Salt solution in small quantities, 5 c.c., is then added at short intervals until the volume of fluid is equal to about 50 c.c. The liquid is drawn off, centrifugated, the supernated fluid poured into a sterile bottle or other suitable receptacle, and the solid portion, which still contains large numbers of yeast cells, is returned to the mill and reground. This process is repeated until microscopical examination fails to reveal unbroken cells in the centrifugated sediment. The various fluid portions are added together and the total volume made up to such a point that every 100 c.c. represents one gram of the dried organism. As a preservative, 0.5 per cent carbolic acid or 0.3 per cent chloroform is then added. As a further precaution the "extract" is kept in the ice-box. If one wishes a fresh extract the moist organisms may be used. It has been shown by experiment that the yeast loses about $\frac{1}{12}$ of its weight when dried. Therefore if one grinds 12 grams of fresh, moist organisms and wishes to make a 1 per cent solution comparable to the preserved extracts, he makes the total volume of extract 100 c.c. This, of course, is not a method of great accuracy, owing to the variations in the amount of water which the moist organisms contain, but it seems fairly satisfactory.

Upon standing any length of time a white, amorphous precipitate separates out of the extract and settles to the bottom. It does not go into solution again when mixed with the overlying fluid. The extract itself has a yellowish, opalescent appearance, which tends to clear somewhat on shaking with ether. It possesses a markedly yeasty smell. The following rough qualitative analysis was made on "Ext. BlR 1/19/09." The results are typical of those observed in all cases examined.

To 10 c.c. of the extract were added 30 c.c. of absolute alcohol, and the mixture was allowed to stand in the ice-box for 30 minutes. At the expiration of that time, a fairly heavy, whitish, flocculent preciptate had formed, which showed a tendency to remain suspended in the liquid in large and small loose clumps. The material was filtered. The filtrate was evaporated to dryness at a temperature not over 65° C. and yielded a yellow, gummy-looking material which possessed a marked sweetish, yeasty smell. This material was allowed to stand over night at room temperature.

The precipitate on the filter paper was washed with 30 c.c. of distilled water in which it seemed to be readily taken up, giving the water a whitish, cloudy appearance. It had no characteristic odor. Under the microscope, the aqueous solution showed a fine, granular débris, but no crystalline suspension; the residue from the evaporated alcoholic extract was amorphous and of a yellow tinge.

The aqueous solution was tested for dextrin, starch, and reducing sugars with negative results. The protein color and coagulation tests resulted as follows:

Heller's nitric acid test	negative
Boiling with glacial acetic	"
Millon's reagent	"
Xanthroproteic test	"
Adamkiewicz	+very sharp
Biuret	? very slight if any
Liebermann	negative
Hopkins-Cole	+very sharp
H_2SO_4 (conc.)+sugar	negative

Similar tests applied to the whole extract gave similar results.

The yellow residue from the alcoholic extract was soluble in ether and chloroform and was not precipitated from the chloroform solution by acetone. It gave a negative Salkowski's test for cholesterol. Further analysis was not attempted.

No experiments were undertaken with killed organisms, the living organisms and the extract alone being used in the immunological work.

As a matter of routine, injections of living organisms were made into the peritoneal cavity; the pleural cavity was used a few times. Intravenous injections are inconvenient in the guinea-pig, while subcutaneous injections are open to the objection that the resulting subcutaneous nodules early cause necrosis and sloughing of the overlying skin, thus not only giving splendid opportunities for secondary infections but tending to scatter virulent mold fungi about the cages, which was regarded as dangerous.

The extract was injected subcutaneously or intraperitoneally according to the seeming needs of the particular experiment in which it was being used.

OIDIOMYCOSIS (BLASTOMYCOSIS) IN THE GUINEA-PIG.

Dosage.—In a series of experiments carried out about three years previously with this same organism, Dr. Ricketts had determined roughly its virulence for guinea-pigs. He found that 0.3 of a gram of an agar plate culture, grown at incubator temperature, injected into the peritoneal cavity of a 375–400 gm. guinea-pig

would cause death in about 35 days, with generalized oidiomycosis, especially in the abdominal and thoracic viscera.

In view of the prolonged course of the disease, even following the injection of such large doses as that mentioned, it did not seem so essential for present purposes to determine a uniformly fatal dose as to find out what dosage would give a constant clinical picture, and, at the same time, would not require a month or six weeks for development. With this in view a series of 42 guinea-pigs were injected with quantities of moist organisms from agar culture, the dosage varying from 0.5 to 0.0001 of a gram. Each pig was examined daily with reference to weight, temperature, and to changes which might be revealed by inspection or palpation. The following general conclusion was drawn: There is no constant symptom by means of which one may diagnose oidiomycosis in the guinea-pig during life. The weight is an unreliable guide, it varies with the abundance of food and the length of time which elapses between feeding and the taking of weights. Again, even if the animal develops an apparently fatal infection, the weight before death may approximately equal that before inoculation, or, if it does decrease, the major portion of the loss occurs during the last week or so of life. The temperature is also unsatisfactory. In the majority of cases when the oidiomycosis cannot be doubted, the temperature runs an absolutely normal course. When it does follow an abnormally high curve, it is difficult to exclude secondary infection, or the possible effect of occlusion of excretory ducts (that is, ureter, seminal vessels, urethra).

If one compares the effects in males and females, however, he finds that there is, symptomatically at least, one marked difference. In males with doses as low as 0.001 gm. one usually can detect small nodules (0.5–2 mm. in diameter) in the testicles within seven to ten days after inoculation; nodules can practically always be found with doses of 0.01 gm. within five to seven days. With doses of 0.1 gm. or over, nodules not only occur regularly but they are of good size and the infection may have a fatal outcome. In the female pig there is absolutely no sure way of diagnosing oidiomycosis during life except to open the abdominal cavity and inspect the contents. One might think of deep pal-

pation, but how distinguish nodules from feces? We have found it practically impossible in most cases. Of great assistance in determining the severity of the infection—and this may be applied to both sexes alike—is the "look" and "feel" of the animals. By the term "feel" well is meant that when the animal is handled the muscles are found to have their normal firmness and tone. In fresh guinea-pigs which have not been handled, the tense, hard, wiry feeling of the muscles is very noticeable. After the animals have been manipulated daily for a week or so, they evidently become used to it and relax readily when they are picked up. In this state the muscles are soft and pliable but have not lost their tone. If such pigs become the victims of a chronic wasting disease, this normal muscular tone is lost; the muscles become decidedly flabby to the touch so that the animal feels like nothing so much as the time-honored "dish-rag." This "feel" may be present before the animal's weight has fallen off appreciably. The animal usually appears thin and we say it looks sick, but this appearance is due more to the staring coat and the "hunched-up" attitude which the animal assumes than to a real emaciation. These symptoms, of course, are characteristic of cachectic conditions in general and are not specific. It should be mentioned that the development of nodules at the point of inoculation noted in some of the pigs was regarded as the result of faulty technic and could be avoided to a considerable extent by rinsing the needle in water before making the injection.

For the remainder of this work, in view of the above facts, the taking of temperatures, the recording of weights, and the use of female guinea-pigs were eliminated excepting in special cases. Male pigs were used as a matter of routine and their condition was adjudged by the results of careful palpation of the testicles and by their general "look" and "feel." 0.1 gm. of moist agar culture was adopted as the standard, surely infective dose.

The difference in the results of intraperitoneal inoculation in male and female guinea-pigs is very marked from the symptomatological standpoint. Take guinea-pig 75 and guinea-pig 81, for example. Each received the same dose from the same culture on the same day. Guinea-pig 75 (male) became weak and flabby

to the touch with a staring coat and enormously swollen scrotum which finally ulcerated. Guinea-pig 81 grew fat and sleek and it was only at autopsy that signs of the infection could be demonstrated. Some of the possible reasons for this difference will be discussed later as will also the question of localization of infection, and, in a general way, the course of the disease and possible treatment.

ACTIVE IMMUNIZATION.

In the endeavor to establish an active immunity in guinea-pigs, use was made of the living organism and of the extract. In the case of the living organism, the method pursued for the greater part of the time was to reinoculate a pig as soon as he had made an apparently complete recovery from his previous infection, and then to study the development of the new infection as regards (1) the time of the appearance of nodules in the testicles, their size, consistency, and the rate at which they disappeared, (2) the development of palpable nodules in the abdominal cavity, and (3) the "look" and "feel" of the animal. The results of such observations had led to the belief that it made practically no difference how many times an animal was subjected to oidiomycetic infection and recovered, he always retained his original susceptibility. In order to put this conclusion to a thorough test the following experiment was performed. All the living pigs which had recovered from previous injections were gathered together with ten normal pigs of about the same average size and all were given an equal dose of living oidiomycetes (approximately 0.1 gm.) and the results carefully watched. The previous history of the so-called "immune" animals is given in Table 1.

On comparing the results of the inoculation in the immune and the control guinea-pigs, some slight, but quite well marked differences were noted. First, regarding the course of the infection, it is a fact worthy of remark, that in so far as the male pigs of the two series are concerned, the immune animals made a decidedly more rapid recovery than the controls, as judged by the rate of disappearance of the nodule from the testicles. The female pigs offer no accessible basis for comparison. If we examine the

temperature charts of the immune and normal animals before and after injection we again find differences. The average temperature for the immunized animals for the three days prior to the inoculation on which temperatures were observed, that is, the 19th, 20th and 23d, is 103.29°+ F.; that for the control animals is 103.31°+ F. The average of the observed temperatures of the immunized animals on the days immediately following the injection (the 24th, 25th, and 27th) is 104.27°+ F.; that of the control

TABLE 1.

HISTORY OF "IMMUNE" GUINEA-PIGS.

GUINEA-PIG NO.	NO. TIMES PREVIOUSLY INOCULATED	AVERAGE INTERVAL BETWEEN INOCULATIONS	TIME ELAPSED SINCE LAST INOCULATED	DOSAGE (IN GM.)			
				1st Dose	2d Dose	3d Dose	4th Dose
1.....	3	61 days	68 days	0.1	0.1	0.1	...
72.....	4	24 "	68 "	0.005	0.1	0.1	0.1
75.....	1	..	166 "	0.5	...	...	..
77.....	4	24 "	68 "	0.001	0.1	0.1	0.1
80.....	4	24 "	68 "	0.0001	0.1	0.1	0.1
81.....	3	33 "	68 "	0.5	0.1	0.1	...
83.....	4	24 "	68 "	0.1	0.5	0.1	0.1
92.....	2	29 "	68 "	0.1	0.1	...	...
47.....	2	24 "	240 "	0.1	0.1	...	...
93.....	2	25 "	50 "	0.1	0.1	...	...
94.....	2	25 "	50 "	0.1	0.1	...	...
95.....	2	25 "	50 "	0.1	0.1	...	...
69.....	3	32 "	68 "	0.1	0.1	0.1	...

guinea-pigs for the same period is 103.49°+ F., or practically 104.3° F. in the one case and 103.5° in the other—a difference of nearly 1° F. To be sure, this difference is small, but in view of the fact that the temperatures of the two groups of pigs were almost identical before inoculation, while following the inoculation the rise was confined almost entirely to one group, it would seem that one would be justified in considering such a rise a positive reaction of the animal organism against the injected oidiomycetes. Apparently, then, a low grade of immunity is developed in guinea-pigs by the intraperitoneal inoculation of living organisms. As symptomatological evidence of the immunity—which seems to be emphatically a relative immunity—we have the more rapid disappearance of the lesions following infective doses and a small but fairly decided rise in temperature on the days immediately following the inoculation. These reactions may be manifest as long as 240 days after the last inoculation (guinea-pig 49)

or, allowing the animal 40 days in which to recover from the immunizing inoculation, a liberal margin, the acquired power to react against renewed infection in a guinea-pig which has recovered from two moderate injections may persist at least 200 days.

A large series of guinea-pigs were given repeated injections of the extract. Of this series but four pigs were tested by the injection of living organisms. Their histories follow:

TABLE 2.

No. of Guinea-Pig	No. of Injections	Interval between First and Last Injection	Interval between Last Injection and Day Tested	Average Volume of Injection	Route
13	23	178 days	63 days	About 1.5 c.c.	Intraperitoneal
37	14	105 "	77 "	2 c.c.	"
38	14	105 "	77 "	2 "	"
56	12	91 "	63 "	2 "	"

Each of these four pigs, together with each of seven control animals, was given 0.1 gm. BlR, intraperitoneally. All of the control animals developed severe infections, large nodules in the testicles, weakness, staring coat, etc.; and one of them died, post-mortem examination revealing generalized oidiomycosis. Of the immune animals, but one developed testicular lesions—a small, solitary nodule; all presented rather large, fairly hard, intra-abdominal masses, but no other symptoms. The subsequent history of the immune pigs follows. Guinea-pig 13 was bled to death on December 3, 1909, 102 days after injection. At autopsy the seminal vessels and portions of the small intestines were found adherent to a fibrous nodule on the anterior abdominal wall, within which was fluid detritus containing oidiomycetic forms; cultures were negative. Microscopic sections show oidiomycetes. No other abnormalities.

Guinea-pig 37 died January 19, 1910, 149 days after injection. At autopsy a mass of dense fibrous adhesions were found about the point of inoculation. In the center of this mass was a cavity 1 cm. in length and 0.5 cm. in width and about 1 to 2 mm. deep in which was fluid detritus. Testicles were normal. No oidiomycetes were found, microscopically, in the contents of the nodule. The cause of death was pneumonia and pericarditis. No oidiomycetes were recovered in cultures.

Guinea-pig 38 was found dead on March 14, 1910, 203 days after injection. Autopsy showed pneumonia and old fibrous adhesions about the point of injection. Testicles were normal.

Guinea-pig 56 was killed March 14, 1910, 203 days after injection. Autopsy disclosed an oval fibrous sac about 1 cm. in greatest length attached to the anterior abdominal wall. Adherent to the sac were the seminal vesicles and portions of the small intestines. Within the sac was fluid detritus; microscopically forms very similar to oidiomycetes were observed; unfortunately these bodies were soluble in ether. Cultures were negative as regards oidiomycetes.

One cannot argue from these experiments that the prolonged immunization with the "extract" resulted in the development of such a high grade of immunity as would lead to the absolute walling off of infective doses of organisms at or near the point of inoculation, for it is not at all certain that those inoculations may not have been made into previously existing adhesions, resulting from the numerous immunizing injections, which could not be detected by external examination. On the other hand, in view of the fact that most of the control animals developed marked infection, one of them dying, and that the nodules which appeared in the testicles of one of the immune animals vanished with considerable celerity, it seems justifiable to assume that a certain degree of immunity, at least, was manifested. Another point of interest which will be considered later was the finding of morphologically typical oidiomycetes in stained sections from guinea-pig 13. Apparently this pig's immunity, whatever it may have been, was not of a markedly lytic type.

EXPERIMENT TO TEST THE EFFECT OF THE INJECTION OF "EXTRACT" INTO INFECTED ANIMALS.

Eggers has shown (*vide infra*) that repeated intraperitoneal injections of oidiomycetic extract seem to decrease the resistance of guinea-pigs to accidental infection. He has also mentioned the fact that the animals so treated suffer a considerable loss of weight. It has been noticed in the present experiments that this loss of weight in guinea-pigs receiving 2 c.c. intraperitoneally

every fourth day may be extreme, the animals become flabby to the touch and appear markedly cachectic. They slowly recover when the injections are discontinued. Bearing in mind the above findings, it became of interest to test infected animals with graduated doses of the extract. The results, as shown in the following experiments, appear to be of possible importance.

A series of 10 guinea-pigs which had recovered from spotted fever eight months to a year before were given intraperitoneal injections of 0.1 gm. of oidiomycetes from an agar slant culture on January 21, 1909. As soon as nodules became palpable in the testicles, five of these pigs were given subcutaneous injections of extract ("BlR 1/19/09," $CHCl_3$ preservative) as indicated below, the other five animals being used as controls.

Guinea-pig 45: January 21, 1909, control pig; wt.=680 gm. 0.1 gm. intraperitoneally; January 25, nodules palpable in testicles; February 8, nodules in testicles about 1 cm. in diameter; March 1, testicles normal.

Guinea-pig 46: January 21, 1909, control pig; wt.=775 gm. 0.1 gm. intraperitoneally. Result like guinea-pig 45.

Guinea-pig 47: control pig; wt.=515 gm.; January 25, slight induration of testicles; February 8, same; true nodules never developed; March 1, testicles normal.

Guinea-pig 48: control pig; wt.=515 gm.; January 25, slight induration of testicles; February 8, testicles contain nodules, some of which are about 1 cm. in diameter; March 1, testicles normal.

Guinea-pig 49: control pig; wt.=955 gm.; January 25, small nodules can be felt in the testicles; February 8, large nodules are palpable in the testicles; March 1, testicles normal.

Guinea-pig 50: January 21, 1909, wt.=690 gm. 0.1 gm. oidiomycetes intraperitoneally. Received subcutaneous injection of 0.5 c.c. extract January 25, 30, February 4, 9, 15, 23; January 25, nodules palpable in testicles. Small nodule at point of inoculation drained and sterilized with 95 per cent carbolic acid and absolute alcohol; wound healed rapidly. Pus contained large numbers of oidiomycetes. February 8, small nodules in testicles; February 28, animal fully recovered.

Guinea-pig 51: January 21, 1909, wt.=680 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 30, February 4, 9, 15, and 23; January 25, nodules in testicles; February 8, nodules in testicles nearly as large as those in control pigs; March 1, animal fully recovered.

Guinea-pig 52: January 21, 1909, wt.=690 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 27, 29, February 1, 3, 5, 8, 10, 12, 15, 17, 23, and 25; January 25, slight hardening of tip of right testicle noted; February 8, nodules in testicles fully as large as the largest among the control pigs; February 25, on this date the nodules had almost disappeared so that the injections were discontinued.

Guinea-pig 53: January 21, 1909, wt.=725 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 27, and 29;

January 25, nodules palpable in testicles; February 1, found dead. Autopsy: The abdominal parietes, testicles, seminal vesicles, and diaphragm are peppered with nodules varying in diameter from 0.5 mm. to 4 mm. A few similar nodules appear on the surfaces of the spleen and liver. In the great omentum along the greater curvature of the stomach is a thick fibrinous mass; a mass of cheesy pus 1 cm. in diameter and 0.5 cm. in thickness surrounded by a hyperaemic zone occurs on the anterior abdominal wall at the point of inoculation. A few grayish patches, 1 to 3 mm. in greatest width, appear beneath the epicardium and seem to involve the heart muscles. The lungs do not appear to be so collapsible as normal; they appear to be congested; no gross nodules. Cultures from the various nodules show oidiomycetes in pure culture. Histological examination of the lungs shows that there is no edema nor exudation.

Guinea-pig 54: January 21, 1909, wt.=610 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously February 1, 3, 5, 8, 10, and 12; February 1, nodules palpable in testicles; February 8, fairly large nodules in testicles; February 15, found dead. Autopsy: The right testicle is transformed into a sac of pus containing oidiomycetes in large numbers. Scattered nodules 1 to 5 mm. in diameter are found on liver, spleen, mesentery, left testicle, and abdominal wall. A nodule in the intermuscular fascia of the anterior abdominal wall is 1 cm. in diameter and 2 to 3 mm. in thickness, and contains an enormous number of budding oidiomycetes. The left lung is very much enlarged and occupies three-fourths of the thoracic cavity; it is hard, non-collapsible, dark in color, with hemorrhagic spots. Heart and right lung appear normal. Material from various nodules smeared on glucose agar slants yielded oidiomycetes in pure culture. Histologically the left lung appears to be in a state of pneumonic consolidation.

This experiment suggests that in the extract we may have a means of modifying to a large extent the course of an oidiomycetic infection in guinea-pigs. Those animals which received 0.5 c.c. of the extract subcutaneously every fifth day seemed to suffer a milder infection than the control pigs and to make, possibly, a somewhat more speedy recovery; of those animals receiving 0.5 c.c. extract subcutaneously every second day, one—guinea-pig 42—paralleled very closely the control pigs in this infection; guinea-pig 53 died in 11 days of generalized oidiomycosis, and guinea-pig 54 died 25 days after injection, autopsy showing extensive oidiomycosis of the abdominal parietes and viscera with a terminal lobar pneumonia; 0.5 c.c. of the extract injected subcutaneously every second day into infected guinea-pigs may be toxic; 0.5 c.c. of the extract injected subcutaneously every fifth day into similarly infected guinea-pigs apparently is not toxic and seemingly increases the animal's resistance to the disease. Broad generalizations from a single experiment such as this are, of course,

unwarranted, but it seems possible that further work along the lines indicated by these results may lead to the development of a technic which will enable us to treat effectively oidiomycetic infections.

EXPERIMENTS IN ANAPHYLAXIS.

In testing further the properties of the "extract," an attempt was made to elicit with it the phenomenon of anaphylaxis in guinea-pigs. It was with such an end in view that the following experiments were performed. The temperature of the animals was observed only in cases where specific mention of that fact is made.

Experiment 1.—Guinea-pig 1 received an intraperitoneal injection of 1 c.c. of "Extract BlH_2," which had been preserved in 0.5 per cent phenol for about 13 months, on October 27, 1908. Sixteen days later 5 c.c. of the "extract" were injected intraperitoneally. Result: no convulsions nor tremors of any kind; pig alive and well 24 hours later.

Experiment 2.—Guinea-pig 17 was treated with doses of "Extract BlH_2," similar to those given guinea-pig 1. An interval of 27 days separated the two injections. Results: Negative, similar to Experiment 1.

Experiment 3.—Guinea-pig 17 received an intraperitoneal injection of 0.05 gm. of organism BlR on November 12, 1908. Twelve days later the animal was found to have lost 90 gm. in weight; both testicles had become swollen and hard. Five c.c. of "Extract BlH_2" were injected intraperitoneally. There ensued a temporary fall of about 8° F. in temperature which returned to normal within 24 hours. No further symptoms appeared. The animal thereafter gained weight.

Experiment 4.—Guinea-pig 31 was given 5 c.c. of centrifugated "Extract BlR" (preservative 0.5 per cent phenol) intraperitoneally on January 4, 1909. The injection was repeated on January 15, without the production of symptoms.

Experiment 5.—Guinea-pig 40 received 0.2 c.c. "Ext. BlR" (preservative 0.5 per cent phenol) intraperitoneally on January 15, 1909. Guinea-pig 41 received 0.5 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 42 received 1.0 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 43 received 1.5 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 44 received 2.0 c.c. "Ext. BlR" intraperitoneally on January 15. On January 26—interval of 11 days—guinea-pigs 40 and 43 received 5 c.c. of Ext. BlR intraperitoneally—no reaction. On January 28 guinea-pig 41 and guinea-pig 42 with a normal control pig received 7 c.c. Ext. BlR intraperitoneally. Negative results. Guinea-pig 44 died of pneumonia before he was tested.

Experiment 6.—Guinea-pig 55, wt. 660 gm., received 0.1 c.c. Ext. BlR (preservative $CHCL_3$ 0.3 per cent) intraperitoneally January 22, 1909. Guinea-pig 56, wt. 795 gm., received 0.7 c.c. Ext. BlR intraperitoneally. Guinea-pig 57, wt. 830 gm., received 1.5 c.c. Ext. BlR intraperitoneally. Guinea-pig 58, wt. 680 gm., received 3.0 c.c. Ext. BlR intraperitoneally. After an interval of 14 days, each of these four animals and each of two normal control pigs were given an

intraperitoneal injection of 5 c.c. of the extract. Only one animal presented any untoward symptoms and one of the control animals died in about 12 hours. Postmortem examination revealed an acute fibrinous peritonitis.

Experiment 7.—Each of three small guinea-pigs averaging about 250–300 gm. in weight was given 0.3 c.c. of Ext. BlR intraperitoneally on February 26, 1909. The chloroform used as a preservative had been allowed to evaporate before the extract was used. After an interval of 17 days each of the above animals and two normal pigs of similar size received 5 c.c. of chloroform free extract intraperitoneally. All of the animals appeared to be sick immediately following the injection. The three sensitized pigs were found dead next morning. One of the control animals died a week later without apparent cause; the other remained in normal condition.

Experiment 8.—A couple of grams of moist oidiomycetes were ground in the porcelain-ball mill for three hours, and then taken up with NaCl solution (0.85 per cent). Four c.c. of this fresh extract were injected into each of two medium sized guinea-pigs. Twenty-six days later, each of these two animals and two normal pigs were given 10 c.c. of a fresh extract intraperitoneally. The two sensitized animals were found dead the following morning; one control pig died two days later, the other a week later. This extract yielded the usual white, flocculent precipitate upon the addition of absolute alcohol.

Experiment 9.—Fresh moist organisms were ground in a mortar with sterile sand in 0.85 per cent NaCl solution containing 0.2 per cent of normal NaOH. The total volume of the ground material was made up to 25 c.c. by the addition of the salt solution containing 0.2 per cent of normal NaOH. Five c.c. of the total was sediment. The fluid, when injected, was yellowish white and opaque. Guinea-pig (*a*): wt. 140 gm., received 0.1 c.c. intraperitoneally. Guinea-pig (*b*): wt. 215 gm., received 0.3 c.c. intraperitoneally. Guinea-pig (*c*): wt. 225 gm., received 0.5 c.c. intraperitoneally.

Eighteen days later guinea-pig (*b*) and a normal guinea-pig of similar size were given an intraperitoneal injection of the original fresh extract, which had been kept in the ice-box, warmed to 37° C. The extract at this time yielded the usual precipitate with absolute alcohol.

Immune Pig (*b*): Temp. before injection, 10:30 A.M., 102° F. Injection 10:50 A.M.
Normal Pig: " " " " " 100.6° F. " " "
Immune Pig (*b*): Temp. 10:55, 100.2° F.
Normal Pig: " " 99.8° F.

12:15	1:45	2:45	3:45	4:45	5:45	10:30 A.M.
99.8° F.	98.2° F.	97° F.	97.6° F.	98° F.	99.2° F.	102.6° F.
96.8° F.	97.6° F.	96.8° F.	97.3° F.	98.2° F.	98.1° F.	100.6° F.

Two days after this, an interval of 20 days after sensitization, guinea-pigs (*a*) and (*c*) and each of two control animals of a size approximating that of pigs (*a*) and (*c*) were given intraperitoneally 5 c.c. of a freshly prepared extract, warmed to 37° C., similar to that originally employed. Injection at 12:20 P.M.

	Temp. at 11:50	12:45	1:50	2:30	4:35	11:00 A.M.
Guinea-Pig (*a*):	102° F.	101.4° F.	98.8° F.	94.4° F.	96.4° F.	102° F.
Control:	100.4° F.	94° F.	96.4° F.	95.4° F.	96° F.	102° F.
Guinea-Pig (*c*):	101.0° F.	100.4° F.	97.8° F.	94.2° F.	94° F.	104° F.
Control:	101° F.	100.2° F.	96.8° F.	94.6° F.	94.4° F.	100.8° F.

In addition to the fall in temperature, pigs (*b*) and (*c*), and, to a lesser extent, (*a*), developed a tense, swollen, and apparently painful abdomen which subsided as the temperature rose to normal. There was no suggestion of such a condition among the control pigs.

From the above experiments, the conclusions seem justified that, (1) the extract is toxic alike for normal, sensitized, and infected animals as indicated by the sharp fall in temperature following intraperitoneal injection of considerable quantities. (2) It seems possible to demonstrate the phenomenon of anaphylaxis by the use of freshly prepared extract, or of extract freed from its preservative ($CHCl_3$), the reaction manifesting itself either by the death of the animal within 12 to 20 hours, or in the development of a sharp intraperitoneal reaction within two or three hours following the intoxicating injection, which subsides within 24 hours. Large doses may kill the control animals; in such cases, the death of the sensitized animals usually precedes that of the normal animals.

PASSIVE IMMUNIZATION.

In view of the seemingly low grade of active immunity which guinea-pigs developed against oidiomycetes, it appeared that efforts to confer a passive immunity could be more profitably expended along other lines so that no such experiments were undertaken. In Dr. H. T. Ricketts' notes on his work on oidiomycosis, which he kindly placed at my disposal, the following experiment is reported:

Rabbit 44, was immunized against BlR as follows:

November 19, 1905, 1 tube of glucose-agar culture (killed) subcutaneously. Local abscess produced.

November 26, 1905, 1 tube of glucose-agar culture (killed) intraperitoneally.

December 4, 1905, 1½ " " " " " " "

December 12, 1905, 1½ " " " " " " "

December 19, 1905, 1½ " " " " " " "

December 26, 1905, 1½ " " " " " " "

January 7, 1906, 2 " " " " " " "

January 17, 1906, 2 " " " " " " "

January 23, 1906, 1 gram living agar culture intraperitoneally.

February 9, 1906, 1 " " " " "

February 16, 1906, 12 c.c. of blood drawn. This blood was defibrinated, centrifugated, and the serum used in the following experiment:

Experiment 10.—February 16, 1906. Weighed amounts of moist agar culture were mixed with varying quantities of "immune" rabbit serum and injected into the peritoneal cavity of a series of guinea-pigs. Control experiments were carried out with normal rabbit serum and with the untreated organisms.

TABLE 3.

	Date	Wt. in Grams	Result
Guinea-pig 1 received 0.3 gm. Bl + 2 c.c. im. serum, intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/24/06	390 395 360 270	Result: Death on 3/24 from generalized oidiomycosis; interval of 36 days. Total loss of wt. = 120 gm.
Guinea-pig 2 received 0.3 gm. Bl+1 c.c. im. ser.+1 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/24/06	375 370 340 245	Result: Death on 3/24 from generalized oidiomycosis; interval of 36 days. Total loss of weight =130 gm.
Guinea-pig 3 received 0.3 gm. Bl+0.5 c.c. im. ser.+1.5 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/23/06	378 350 285 ?	Result: Death on 3/23, from generalized oidiomycosis; interval of 35 days.
Guinea-pig 4 received 0.3 gm. Bl+2 c.c. normal serum, intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/11/06	400 400 ?	Result: Death on 3/11, from generalized oidiomycosis; interval of 23 days.
Guinea-pig 5 received 0.3 gm. Bl+1 c.c. normal serum+1 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/18/06	400 375 270	Result: Death on 3/18 from generalized oidiomycosis; interval of 30 days. Total loss of weight =130 gm.
Guinea-pig 6 received 0.3 gm. Bl+0.5 c.c. normal serum+1.5 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06	425 410 380	Result: Animal lost.
Guinea-pig 7 received 0.3 gm. Bl+2 c.c. NaCl sol. (0.85 per cent) intraperitoneally 2/16.	2/16/06 3/ 7/06 3/16/06 3/29/06	386 365 325 230	Result: Death from generalized oidiomycosis on 3/29. Interval of 41 days. Total loss of weight =156 gm.
Guinea-pig 8 received dose similar to that of guinea-pig 7.	2/16/06	390	Result: Accidentally killed 2/22/06, six days after injection.

One cannot argue from these results that the "immune" serum conferred an immunity of any degree. It should be remembered in this connection, however, that rabbit serum is somewhat toxic for guinea-pig leukocytes, a property which might mask the protective power of such a serum. There seems to be a suggestion of a reaction of this kind in the results of the above experiment, but more work must be done before conclusions may be drawn.

IMMUNOLOGICAL REACTIONS.

Some preliminary work along this line, carried out with the organism "BlR," was done in 1905 by Dr. H. T. Ricketts, and continued during the year 1906–7 by Dr. H. E. Eggers. Dr. Ricketts performed the following experiment: A guinea-pig was immunized against the oidiomycetes by the repeated injection of

killed organisms and of extract as is shown in the accompanying table.

GUINEA-PIG 1.

Date	Weight	Inoculated with
February 7		0.1 gm. killed agar culture intraperitoneally
February 16		0.1 " " " " "
February 27		0.1 " " " " "
March 9		2 c.c. of extract subcutaneously
March 16	390 gm.	2 " " " "
March 25		2 " " " "
March 30	315 "	Killed on account of loss of weight, and serum used for precipitation tests.

PRECIPITATION TESTS. APRIL 4, 1906. BLASTOMYCES R—EXTRACT II. SERUM FROM IMMUNIZED GUINEA-PIG 1.

I. Original Extract II, after prolonged centrifugation to remove all sediment, was used as antigen. The fluid was whitish-opalescent.

Extract	Immune Serum	Normal Serum	NaCl (0.85 per cent) Sol.	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	Distinct precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Marked "
0.5 "	0.3 "	0.0 "	0.2 "	Fairly heavy "
0.5 "	0.0 "	0.1 "	0.4 "	No "
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

II. Absolute alcohol precipitate of Extract II, freed of alcohol and redissolved in NaCl (0.85 per cent) solution. Fluid was slightly opalescent but much less so than the centrifugated extract.

Redissolved Precipitate	Immune Serum	Normal Serum	NaCl Sol. (0.85 per cent)	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	Distinct precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Marked "
0.5 "	0.3 "	0.0 "	0.2 "	Fairly heavy "
0.5 "	0.0 "	0.1 "	0.4 "	No "
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

III. Clear, colorless, non-opalescent filtrate of Extract II used as precipitogen.

Filtrate	Immune Serum	Normal Serum	NaCl Sol. (0.85 per cent)	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	No precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Very faint precipitate
0.5 "	0.3 "	0.0 "	0.2 "	Small, but distinct precip.
0.5 "	0.0 "	0.1 "	0.4 "	No precipitate
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

Normal serum alone = no precipitate.
Immune serum alone = no precipitate.
Centrifugated extract alone = no precipitate.
Redissolved alcoholic precipitate alone = no precipitate.
Filtrate alone = no precipitate.

The records of work done by Dr. H. S. Eggers were lost before the present work was begun. Dr. Eggers has, however, written a summary of his work from memory which is introduced at this point with his permission:

The following work on attempted immunization to blastomycosis was carried on, on guinea-pigs, rabbits, and goats.

The plan was followed of injecting into the animals, at periods of from seven to eight days, an extract prepared from the dried blastomyces by grinding. Before injection care was taken to shake the material thoroughly to secure suspension of the solid fragments of the organisms.

Using this suspension, work was begun on 12 guinea-pigs and 9 rabbits. 0.5 c.c. of the material was injected into the guinea-pigs, 1 c.c. into the rabbits. Injections were for the most part intraperitoneal; they were repeated every seven to eight days. The condition of the animals was controlled by weighing. If any considerable loss of weight followed an injection, one, occasionally more, periods were passed over without injection. In the case of some of the animals a sharp reaction followed the first few injections; several died within two or three days. Postmortem examination revealed acute parenchymatous changes, particularly in the liver and kidneys. Other animals were substituted for the ones so lost. Several of the rabbits and guinea-pigs became pregnant during the course of the work; such animals were injected subcutaneously while pregnant.

These injections were carried on from early in October, 1906, until January, 1907. At this time serum from several of the rabbits was tested with the homologous extract for a precipitation reaction. A positive reaction, not given beyond a dilution of 1:20, was found at this time with one animal. The serum of another one, which had shortly previously given birth to young, as well as the serum of the young was tested, both being negative. With the animal showing the slight positive serum reaction the injections were kept up for a month; no increase in reaction resulted, and shortly after this the rabbit died of a meningitis.

The periodic injection was carried on longer, the doses being increased until the guinea-pigs received 1 c.c. at a time and the rabbits two. Evidently their vitality was considerably lowered by the repeated injections, as a considerable number, of the rabbits especially, died of secondary infections—meningeal for the most part. Although the injections of the few remaining rabbits were continued until June, 1907, in no case was I successful in getting a precipitation reaction beyond a dilution of 1:20. The rabbits showing even this reaction were too few to enable any decisive test as to their degree of immunization to be made.

Early in April, 1907, that is, six months after injection had been begun, six of the eight guinea-pigs that had been injected a sufficient number of times to warrant expectation of results were injected intraperitoneally with varying doses of a freshly prepared suspension of blastomyces, the doses being 0.25, 0.5, 1, 2, 3, and 4 c.c. respectively. Six normal animals were injected with similar doses. In no case did any of the animals die with any of the findings of blastomycosis. The general lowered resistance of the previously injected animals was betrayed by the fact that for the most part they were survived by the normal animals.

Late in October, 1906, weekly injections of a goat were begun, the animal receiving at the start 3 c.c. of the extract subcutaneously, later increased gradually to 5 c.c.

The serum of this animal, tested late in January, was found to give a positive precipitin reaction with the homologous extract in a dilution of 1:25. With the extract of another strain of the organisms—"H II"—it gave a precipitate in a dilution of 1:12.5; with the third strain no reaction at all occurred. Injections were continued, until the animal was noticed to be becoming thin and feeble. It finally succumbed late in February; postmortem examination revealed a bronchopneumonia, other tissues negative. The serum at the time of its death reacted in the same manner and in similar dilutions to the results just given.

Work was at once begun with another goat, the injections, of 3 c.c. each, being made subcutaneously at eight day intervals. This was kept up until the beginning of June, three months, at which time precipitin reactions similar in every way to those obtained with the preceding animal were found.

As the formation of antibodies with this method of repeated small injections was evidently rather slight, a second goat was obtained, and injected with larger and ascending doses at somewhat greater intervals, 10 or 11 days. The first dose, of 20 c.c. of extract, given subcutaneously, produced a marked reaction, the animal being quite sick for three or four days afterward. Local reaction at the site of injection was extremely slight. The next dose of 30 c.c. was again followed by a marked reaction, the third dose of 40 c.c. by considerably less.

The same plan was followed on rabbits and guinea-pigs. Before this time the writer left Chicago for the summer, and the injections were made without the possession of facilities for testing the serum reactions of the animals. The change was disastrous to the rabbits, as all of them died of an acute enteric affection. The quantity of extract on hand did not warrant work being begun on new rabbits. The guinea-pigs were injected successively with doses of 1, 3, 5, and 7 c.c. After two doses of this last amount had been given, it was dropped back to 2 c.c. per dose; the dosage of the goat, after reaching 40 c.c., was dropped back to 10. In the case of the guinea-pigs, the reaction from the larger doses used here was no more marked than from all the first few smaller doses used previously.

While out of the city, the first of the two goats, which was still being injected with the repeated small doses of extract, died; postmortem examination showed a condition of apparently long-continued pylorospasm, caused by the lodging of a pin in the gastric mucosa.

The remaining animals, the second goat and the guinea-pigs, were brought back to the city. At this time the writer was obliged to discontinue the work.

In the fall and winter of 1908–9 the work on precipitins was continued, guinea-pigs being used as the experimental animals. The results are tabulated below. Some of the animals were immunized by injections of the extract of another organism than BlR, known in the laboratory as Bl H II.

The experiment of Dr. Ricketts with the centrifugated, filtered, and redissolved alcoholic precipitate of the extract was repeated, using the serum of guinea-pig 5 as the immune serum, with positive results in all cases. Thereafter the centrifugated extract was used exclusively as the precipitogen in the precipitation experiments.

The technic used in the work was as follows: Blood was drawn, usually from the guinea-pig's heart, defibrinated, and centrifugated, and varying quantities of the elcar serum added to a constant amount of centrifugated extract in small test-tubes, and the mixtures made up to a uniform volume by the addition of NaCl solution (0.85 per cent). The tubes were then incubated at 37° C. for two hours, placed in the ice-chest, and results observed after 24 hours. Control tubes of normal serum plus extract in dilutions similar to those of the immune serum, and of immune serum and NaCl solution, normal serum and NaCl solution, and extract plus NaCl solution, were always prepared as a part of each experiment. It was noticed that occasionally after 72 to 96 hours in the ice-chest precipitates formed in tubes, which at the end of 24 hours were negative. As the control tubes remained clear and without sediment, and as no proofs of contamination could be obtained by cultural methods or by direct microscopical examination, it was assumed that such precipitates probably represented a specific reaction and they were therefore noted in subsequent experiments. Once in a while, the serum of normal animals formed a precipitate with the extract. Of the 22 normal animals tested, 2 or approximately 10 per cent gave positive reactions, one in a dilution of 1:3 after 24 hours and the other in a dilution of 1:10 on the 4th day.

In Table 4 the figures 1:7, 1:10, etc., represent the highest dilutions at which precipitates appeared; a dash (—) indicates that no observations were made and "0" indicates absence of precipitate.

Six animals were tested more than once. Their history appears in Table 5.

Of the 32 immune sera tested, 22 reacted positively in from 24 to 96 hours. Nine were absolutely negative in so far as they were observed. Of the sera of those animals which were immunized by the injection of extract alone, 8 reacted positively in 24 hours, 0 in 48 hours, 7 in 72–96 hours in one or another of the tests, and 4 were negative; of the sera of those which received the organisms alone, 4 reacted positively in 24 hours, 0 in 48 hours, 1 in 72–96 hours, and 4 were negative; of the sera of the animals which received both extract and organisms, 2 were positive in 24 hours and 1 was

TABLE 4.
PRECIPITINS.

GUINEA-PIG NO.	NO. INJECTIONS OF		NO. OF DAYS INTERVAL SINCE LAST INJECTION	AVERAGE DOSE C.C.	NO. INJECTION LIVING ORGANISMS	DOSE GM.	NO. OF DAYS INTERVAL SINCE RECOVERY FROM LAST INJECTION	RESULTS		
	Ext. H II	Ext. BIR						24 Hrs.	48 Hrs.	72–96 Hrs.
1.....	0	4	4	2	0	—	—	0	—	—
5*.....	4	0	37	1	0	—	—	1:20	—	—
8.....	9	8	13	H II 1 BIR 2	0	—	—	1:20	—	—
10.....	5	0	28	2	0	—	—	0	—	—
I.....	0	0	—	—	3	0.1	28	0	—	—
17.....	0	0	—	—	1	0.05	about 26	0	0	0
22.....	0	0	—	—	1	0.01	20	0	—	—
31.....	0	2	24	5	0	—	—	0	0	1:20
32.....	0	5	6	2	0	—	—	0	—	—
36.....	0	8	14	2	0	—	—	0	0	1:20
39.....	0	4	11	2	0	—	—	0	—	—
43.....	0	9	15	2	—	—	—	1:20	—	—
49.....	0	0	—	—	2	aver. 0.1	about 19	0	0	1:20
50.....	0	6	119	5 subcu.	1	0.1	119	1:7	1:10	1:20
51.....	0	6	122	5 subcu.	1	0.1	122	1:5	1:10	1:20
52†.....	0	13	133	5 subcu.	1	0.1	about 133	0	0	? (0)
56.....	0	9	48	3	0	—	—	—	—	1:7
57.....	0	9	48	3	0	—	—	0	—	1:10
58.....	0	8	48	3	0	—	—	0	—	1:10
75.....	0	0	—	—	2	1st dose 0.5 2d 0.1	about 60	0	0	0
83.....	0	0	—	—	5	2d 0.5 others 0.1 each	about 60	0	0	0
92.....	0	0	—	—	3	aver. 0.1	"	‡1:10	—	—
95.....	0	0	—	—	3	"	"	‡1:10	—	—
II.....	0	2	28	0.1 5	0	—	—	0	0	0
III.....	0	2	2	1st dose 20 c.c. 2d 10 c.c.	0	—	—	0	—	1:10

* Not tested in higher dilution.
† Blood drawn July 13 but not tested until July 22.
‡ Ext. H_2 used as precipitogen.

TABLE 5.

GUINEA PIG NO.	TOTAL NO. INJECTIONS BEFORE						NO. OF DAYS INTERVAL BETWEEN TIME OF LAST INJECTION OR INFECTION AND			RESULTS		
	1st Test		2d Test		3d Test							
	Ext.	Liv. Orgs.	Ext.	Liv. Orgs.	Ext.	Liv. Orgs.	1st Test	2d Test	3d Test	1st Test	2d Test	3d Test
13*....	17	0	23	0	23	2	13	3	100	1:20	1:20	0
35.....	8	0	14	0	—	—	14	6	—	1:20 after 3 da.	1:5 after 4 da.	—
37.....	8	0	14	0	—	—	11	5	—	1:20	1:1 only after 4 days	—
38.....	8	0	11	0	—	—	13	3	—	0	1:20	—
55.....	12	0	12	0	—	—	6	60	—	1:20	1:5 after 48 hrs.	—
81.....	0	4	0	4	—	—	27	60	—	1:20	1:10	—

*At autopsy of guinea-pig 13 (date of 3d test) oidiomycetes were found in a nodule in the abdominal cavity. Cultures were negative. See p. 204.

negative. There does not seem to be any very constant relation between the number of injections, the interval between the time of the last injection and that of the test, and the results obtained; nor does the quantity of the living organisms introduced seem to be of paramount importance. About all that one seems justified in concluding from the above experiments, is (1) that the serum of some normal guinea-pigs will cause a precipitate when mixed in proper proportions with oidiomycetic extract, and (2) that immunization with the extract, infection with living organisms, or combined infection with oidiomycetes and immunization with extracts results in the development of precipitating substances in the serum of about 70 per cent of the animals so treated (the percentages in the above experiments were: extract alone 79 per cent, organisms alone 50 per cent, organisms plus extract [small quantities were used] 67 per cent). These figures suggest also that living organisms plus small quantities of extract, too small to produce noticeable symptoms themselves, may be much more effective in calling forth antibodies than living organisms alone and so rather tend to support the deductions made from the results of the experiment described on p. 207, according to which it was suggested that the extract may have some value as a curative agent. As a diagnostic procedure, with guinea-pigs at any rate, the precipitation reaction would have to be controlled by symptomatological findings; a negative reaction would mean nothing, a positive reaction would be suggestive but not conclusive because of the fact noted above that normal guinea-pig serum may precipitate with the extract in dilution as high as 1:10, after 96 hours in the ice-chest, at least.

According to Dr. Eggers' work, the results with rabbits are even more variable than with guinea-pigs. The goat seems to give a more constant reaction, but even in that case the highest dilution at which a precipitate formed was 1:25, which is certainly not evidence of a very powerful serum.

AGGLUTININS.

At the same time that the precipitating powers of the various sera were being tested, parallel experiments on their agglutinating

power were attempted. Emulsions of the whole organism in 0.85 per cent NaCl were used in these experiments. The serum dilutions were as follows: For methods (*a*) and (*b*)—see below—whole serum 1 part + emulsion 1 part; 50 per cent serum 1 part + emulsion 1 part; 10 per cent serum 1 part + emulsion 1 part. For method (*c*) the dilutions were:

Serum	Emulsion	NaCl
0.05 c.c.	0.5 c.c.	0.45 c.c.
0.1 "	0.5 "	0.4 "
0.15 "	0.5 "	0.35 "
0.2 "	0.5 "	0.3 "
0.25 "	0.5 "	0.25 "
0.3 "	0.5 "	0.2 "
0.35 "	0.5 "	0.15 "
0.4 "	0.5 "	0.1 "
0.45 "	0.5 "	0.05 "
0.5 "	0.5 "	0.0 "
1.0 "	1. loop of moist organisms	0.0 "

Three different methods were tried: (*a*) hanging drop in a hollow-ground slide, (*b*) quantities of 1 to 2 c.c. in watch glasses, which could be observed with ease both with the unaided eye and with the microscope, and (*c*) the materials were placed in narrow test-tubes with the idea that something might be learned from possible variations in the rate with which the oidiomycetes settled out of the suspension. In each experiment the following mixtures were made: (1) immune serum plus emulsion, (2) normal serum plus emulsion, (3) salt solution plus emulsion. These mixtures were incubated at 37° C. for from 1 to 12 hours and then allowed to stand at room temperature until observations were discontinued.

The results were practically identical with the normal and immune sera in all the experiments. It was found that if the organisms were allowed to stand in contact with the serum, normal or immune, for a few hours, a beautiful agglutination reaction, as judged by the naked eye, occurred. This is especially noticeable in the watch-glass preparations. Under the microscope, however, the clumps which had attracted the attention resolved themselves not into masses of agglutinated oidiomycetes, but into groups of oidiomycetes which had developed hyphae, and it was to the appearance of this mycelial network that the apparent agglutination was due.

Regarding the rate at which the organisms settled out of suspension in the tubes, no differences between the normal and immune sera could be determined. The salt-solution tube sedimented first, the other tubes sedimented at about the same rate, and the organisms, like those used in the other methods, sprouted in a few hours.

It is true that sometimes, especially with the hanging-drop and the watch-glass methods, there appeared to be a tendency for the organisms to gather into a few, large, loosely constructed groups, but this occurred with normal serum as well as immune, and almost as frequently with the salt solution alone, and the question arose whether it might not be the expression of an imperfect trituration of the organism in the preparation of the emulsion. Certainly no specific agglutination of oidiomycetes by "immune" sera which did or did not yield a precipitate with the extract was observed.

Possibly the experiment might be more successful if the organisms were first partially ground and if the fragments thus obtained were used; the spherules described within the conidia and the hyphae might also be used.

LYTIC AND BACTERICIDAL SUBSTANCES.

It had been observed in the agglutination experiments that oidiomycetes were able to develop hyphae when suspended in undiluted or diluted normal and immune serum, and that sometimes organisms which had been subjected to the action of such fluids for three or four days grew when planted on agar. It was therefore argued that neither normal nor immune sera possessed lytic or bactericidal properties. To put the matter to a test a number of experiments, of the following nature, were performed.

The sera of guinea-pigs 11 and 55 (see Tables 1 and 2) were mixed with constant quantities of oidiomycetic emulsion in dilutions similar to those used in method (*c*) of the agglutination tests at the same time that those tests were made. These mixtures were incubated for 30 minutes, placed in the ice-chest over night, and then allowed to stand at room temperature for three days. At the expiration of that time the entire contents of each tube was

transferred to an agar slant, which was kept in a horizontal position for 30 minutes in order to allow the organisms to settle on the agar surface, and then placed in an upright position in the incubator with as little agitation as possible. Observation five days later (June 30, 1909) showed growth on the agar surface in all tubes, both above and beneath the surface of the liquid. The colonies growing beneath the surface were in general much smaller than those above the fluid, and gave the impression that they were growing much more slowly. On July 6 the following notes were made: Heavy growth on agar slope above fluid in all tubes excepting tube containing 0.8 c.c. serum (guinea-pig 55) and tube containing 0.2 c.c. serum (guinea-pig 11); no evidence of further growth beneath the surface of the fluid. In tubes containing 0.3 c.c., 0.6 c.c., 0.7 c.c., and 1.1 c.c. serum of guinea-pig 55 and that containing 0.5 c.c. serum of guinea-pig 11, practically all of the water of condensation and introduced fluid has evaporated. In such cases a heavy growth extends to the bottom of the slope. July 8, no further growth beneath the surface of the fluid in any case; otherwise conditions are unchanged. Growth is abundant in all tubes excepting tubes 0.2 c.c. and 0.5 c.c. (guinea-pig 11) and tubes 0.4 c.c. and 0.8 c.c. (guinea-pig 55), in which the growths are but fair.

The sera of guinea-pigs 50, 51, and 52, together with the sera of three normal pigs, were next tested in a manner similar to the above, with the difference that in these cases the tubes were not placed in the ice-chest, that control tubes of emulsion plus salt solution were run and the mixtures were incubated for 48 hours before being transferred to the agar slope. In these cases the results with normal and immune sera were about the same. In all tubes containing serum, growth was slight or did not occur at all; while in the control salt-solution tubes growth was abundant.

It would seem from these experiments that normal and immune guinea-pig serum may have the power of impairing the vitality of oidiomycetes, as suggested by Gilkinet for beer yeasts when subjected to the action of rabbit serum. However, these experiments are regarded as merely of a preliminary nature, and it is hoped that it may be possible to test the above conclusion thoroughly at a later date.

DEVIATION OF COMPLEMENT.

It cannot as yet be stated positively whether or not normal or immune guinea-pig serum will deviate the complement according to the Bordet-Gengou technic. The few sera which have been tested, six recently immunized and one normal, have given absolutely negative results. The experiments have been carried on side by side with successful efforts to demonstrate a specific antibody in the serum of guinea-pigs immunized to a bacillus of the hog cholera group, so that in one way at least the work has been abundantly controlled. On the other hand, the positive results reported by other workers with various yeasts and molds make it still seem possible that the technic used and not the oidiomycetes may be at fault. One thing which has been noticed is that 0.1 c.c. of a moderately turbid emulsion of oidiomycetes, or 0.1 c.c of a freshly made extract, or 0.15 c.c. of redissolved alcoholic precipitate of the extract, is usually sufficient to inactivate completely the complement in 0.01 c.c. of guinea-pig serum which has stood in the ice-chest over night. This is, of course, but the expression of the property, common to all yeastlike forms, of absorbing or destroying complement. With this fact in mind, however, it is to be regretted that those who have reported positive results with various mold fungi have neglected to give the full protocol of even one experiment. It might be of interest to compare the complement-binding power of various pathogenic and non-pathogenic yeast plants; possibly, by this means, some further idea of the factors which determine their power of resistance to destruction by the animal body might be gained.

CUTANEOUS AND OPHTHALMIC REACTIONS.

From the results of the skin and conjunctival tuberculin tests as applied in man, it appeared of interest to experiment in an analogous way with the oidiomycetic extract on infected guinea-pigs.

Cutaneous reaction.—The method of applying this test was as follows: A space on the abdomen about 3 cm. square was shaved and washed with $HgCl_2$ (1:1000) and alcohol (80 per cent). After drying, four small areas, forming the corners of a square of about 1 cm. in breadth, were scarified with the blade of a blunt knife

which had been dipped into the following materials: for area No. 1, the extract of organism BlH_2; for area No. 2, the extract of organism BlR; for area No. 3, 0.5 per cent carbolic acid. For area No. 4, the dry blade alone was used. 0.5 per cent carbolic acid was applied because the extracts had been preserved in that strength of carbolic acid. In cases in which chloroform had been used as a preservative, a 0.3 per cent aqueous solution of chloroform was substituted for the phenol. In cases in which fresh extract alone was used, the carbolic acid and chloroform controls were omitted.

Experiments on animals suffering from light, moderate, and severe infection resulted uniformly and negatively. It appeared to make no difference at what time with reference to the infection (early, middle, or late) the tests were applied; the results never varied. Repeated scarification of the same areas after intervals of from 3 to 14 days called forth no reaction.

Ophthalmic reaction.—The technic used in these experiments was as follows: To 10 c.c. of the extract was added an equal volume of absolute alcohol. The resulting precipitate was removed by filtration, washed in absolute alcohol, and dried in a partial vacuum over sulphuric acid. The dried material was redissolved in salt solution (0.85 per cent NaCl). A drop of this solution was instilled into the eye of the animal to be tested, the dose being repeated in the same and in the opposite eye after an interval of from 3 to 10 days. The results were always negative. The fresh extract and the extract preserved in phenol and in chloroform were also used, care being taken to control with corresponding aqueous solution of carbolic acid and chloroform, with similar negative results.

Apparently then cutaneous and ophthalmic reactions against the extract do not occur in guinea-pigs infected with oidiomycosis.

THE FATE OF THE OIDIOMYCETES WHEN INJECTED INTO THE PERITONEAL CAVITY OF THE GUINEA-PIG.

Changes in the peritoneal fluids of recently injected animals.—The technic and results of this study are best illustrated by the report of the following typical experiment.

Guinea-pig 86 (normal) was given an intraperitoneal injection of 3 c.c. of a rather dense emulsion in salt solution (0.85 per cent) of a nine day agar culture of oidiomyces at 11:10 A.M., April 8, 1909. By means of a fine glass tube, specimens of peritoneal fluid were drawn off at approximately hourly intervals. Some of this material was placed upon a slide, a cover glass applied, and sealed with paraffin. The specimen was then examined microscopically without further delay. Cover glass smears were made of other portions and stained with the Giemsa mixture. These preparations were examined at leisure. The guinea-pig was kept on his back for a few minutes before fluid was withdrawn, but was allowed to run free in his cage between times.

12:00 M.: Two specimens of clear, slightly viscid fluid were obtained. One, slide 1, was incubated for 30 minutes, the other, slide 2, was examined immediately.

Slide 2: Quite a number of red cells; a very small number of leukocytes, and these mostly mononuclear; no oidiomycetes found; red cells probably due to trauma incident to the insertion of the glass pipette, since this occasioned a slight hemorrhage. Slide 1 is similar to slide 2 in all respects.

Stained smear.—Very few polymorphonuclear leukocytes, about in the same proportion to the number of red cells as in the blood; the vast majority of the leukocytes are mononuclear cells; some are clearly small lymphocytes; most of them are large mononuclear leukocytes, some approaching a distinctly endothelial type as judged by the abundance of their cytoplasm. Many of the leukocytes appear to be disintegrating and losing their staining power. One organism is present. It is free. The cytoplasm takes an irregular bright blue stain; the capsule appears as a bright, sharply outlined, shiny rim, with merely the suggestion of a bluish tinge.

1:20 P.M. Slide 3: Still many red cells; many times more leukocytes, seemingly mostly mononuclears, a few organisms, some apparently free, most of them surrounded by from three to forty leukocytes; in some cases the organism has been ingested by a single leukocyte while the other leukocytes crowd around; in other cases ingestion has seemingly not occurred, though two or three layers of leukocytes inclose the organism. The latter appear to be unchanged.

Stained smear.—Mononuclear cells still predominate, although the proportion of polymorphonuclear cells has increased. The leukocytes are frequently clumped, in some cases about organisms. The leukocytes in these clumps seem to be mostly mononuclears; a few polynuclears occur.

2:20 P.M. Slide 4: There are about the same number of red cells as in slide 3, but many more leukocytes. Leukocytes occur singly and in large and small groups, the tendency being toward the formation of grape-bunch-like masses of cells so large and compact that it is impossible to make out individual cells excepting at the periphery. Sometimes one can distinguish organisms down deep in these masses; frequently cannot make sure of the identity of anything there; no organisms found in very small masses or in single leukocytes. In one instance an oidiomycete can be seen within a single leukocyte, near the periphery of one of the large masses; other organisms are present within that mass, but one cannot tell whether they have actually been ingested or not. No organisms lying free. Exudate is more viscid than before, but does not seem to coagulate readily. The oidia are apparently unchanged.

Stained smear.—The majority of the leukocytes approach the transitional type; there are quite a number of polynuclears; cells grouped about organisms are mostly large mononuclear to transitional in type.

3:20 P.M. Slide 5: Exudate is very abundant and viscid; cloudy and granular.

Microscopically very similar to slide 4, with the exception of the greatly increased number of leukocytes. Many of the organisms can be seen definitely phagocyted as described in 4. All organisms which can be well defined are found to be completely engulfed by one to three leukocytes, generally one, the rest of the leukocytes in the mass simply forming a many layered capsule about the organism and its phagocytes. Exudate does not clot in 30 minutes.

Stained smear.—Large numbers of polynuclear cells, otherwise similar to 4. Cytoplasm of cells grouped about oidiomycetes is fused into a single mass in some cases; in others the cells are simply connected by strands of cytoplasm, giving a vacuolated appearance to the region between the cells.

4:20 P.M. Slide 6: Exudate is abundant. It is still richer in leukocytes than before; there are quite a number of phagocyted organisms. The leukocytes do not seem to be bunched so strikingly as in previous slides; the oidia are inclosed within from 1 to 10 or a dozen leukocytes; the leukocytes are more compact about the organisms than formerly, and instead of appearing like a bunch of grapes, that is, grouped rather loosely about phagocyted organisms, the leukocytic mass now has the appearance of segmenting frog eggs, all of the cells fitting closely to each other. None of the very large bunches of leukocytes are seen which were so striking in slides 3, 4, and 5. Exudate does not coagulate readily.

Stained smear.—Polynuclears predominate largely, and seem to be the phagocytes.

5:20 P.M. Slide 7: Large clumps of leukocytes about oidiomycetes do not appear. Frequently a single organism may be found within one leukocyte; almost never are more than five or six leukocytes concerned, excepting where a number of organisms occur together, in which case the whole mass may be fairly large, though the number of leukocytes per organism is apparently not what it was in earlier slides. The organisms appear to be unchanged.

Stained smear.—Similar to 6. The nuclei of the leukocytes appear to be undergoing a process of karyorrhexis.

6:20 P.M. Slide 8: Similar to the above in the main. One important difference, i.e., leukocytes immediately surrounding organisms seem to be breaking up; they are losing their distinctness of outline and the granules are spreading out diffusely into the surrounding zones. Oidia are all budding, this condition seeming to be a little more marked than in earlier specimens of the exudate.

Stained smear.—Similar to 7, more leukocytic changes. It is hard to determine whether the phagocytic cells are mononuclear or polymorphonuclear; it seems best to regard them as mononuclear, although doubtful.

March 9, 1909. 9:30 A.M. Slide 9: Leukocytes grouped about organisms seem to be fragmenting. Leukocytes are scattered evenly over the field. Aggregations of 20–30 about an organism, especially a budding form, are still to be found; those leukocytes in the center of such masses seem to be more or less fused with each other. Oidiomycetes contain variable numbers, generally inconsiderable (10–20), of small, somewhat highly refractive, spherical bodies, arranged as a rule near the periphery of the cell within the capsule. Frequently they are scattered irregularly through the cell. Many of the leukocytes in diverse parts of the field contain very similar granules. Sometimes organisms appear to be full of such bodies.

Stained smear.—In a number of instances it can be seen that the phagocytosis is undoubtedly by large mononuclear cells. One group is especially noticeable;

it consists of a budding organism inclosed by four leukocytes. The leukocytes are arranged radially about the organism. The peripheral three-quarters of the cytoplasm of each cell is independent and distinct, the inner fourth, however, has merged with a corresponding part of the other three to surround the oidium by a uniform cytoplasmic mass. The disposition of the cells reminds one a little of the structure of a four-leaf clover. The nuclei of the leukocytes stain purple; the cytoplasm, which is moderately vacuolated, a robin's-egg blue. The intracapsular portion of the organisms takes an irregularly distributed, deep to light blue stain; the capsule is clear, homogeneous, and of a very light blue color.

10:30 A.M. Slide 10: Cells, unstained, appear like large mononuclears for the most part. Groups of leukocytes are sometimes found arranged radially about an indistinct, somewhat homogeneous central mass in which it seems one can make out leukocytic shadows. The cells at the periphery of such masses are more or less cone-shaped, apex inward, nucleus at the base, peripheralward. The apex is drawn out into a narrow projection which loses itself in the central area. Budding oidia are found packed full of granules; then again non-budding forms occur which contain a very indistinct, faintly granular, slightly refractive material. Organisms are still engulfed by leukocytes, though it looks sometimes, especially in the case of the budding, strongly granular forms, as though the leukocytes were beginning to break away from the organism and its immediate phagocyte. Again we find budding, markedly granular organisms in the center of such leukocytic masses as are described above. Granules may or may not be present in the buds of such organisms. Phagocyted oidia are observed in which the cytoplasm seems to have become homogeneous and is divided into two or three segments.

Stained smear.—Sometimes the nucleus and cytoplasm of the leukocytes are markedly vacuolated, and the cell appears to be disintegrating. In a few instances the intracapsular portion of the oidia stains much less deeply than usual while the immediate leukocytes are quite well preserved. Polymorphonuclear cells are much more numerous than the other varieties of leukocytes, but appear to take no part in the phagocytosis. Typical small lymphocytes are very few in number. The large mononuclear cells with a great deal of cytoplasm form the great bulk of mononuclear leukocytes.

2:30 P.M. Slide 11: Essentially similar to 10. One organism is present which has an unusually thick capsule (2–3 times ordinary thickness). The outside of the capsule is smooth, the inside irregular. This organism has one small bud. Other oidiomycetes in the same mass appear normal.

Stained smear.—Phagocytosis is not exclusively by mononuclear leukocytes; a few polymorphs may be included in the plasmodial masses.

5:00 P.M. Slide 12: Leukocytic groups occur about organisms as in the two preceding slides. Fusion of leukocytes and extrusion of granules—breaking up of leukocytes?—is, if anything, more marked. As many as seven budding organisms are found in one leukocytic mass. Oidia seem to be budding and multiplying since we now find two, three, or four cells grouped together, sometimes in chains—mother cell, daughter cell?—sometimes in pairs nearly always giving evidence of budding. One organism is found with two buds. Also find free organisms for the first time since the second hour. One of these is rather thick-walled and is packed with 25–30 spherical, homogeneous bodies. The spherules are not so numerous but that each can be distinguished.

Stained smear.—Similar to 11. Phagocytic cells seem to be breaking up.

April 9, 1909. 8:20 A.M. Slide 13: Many free organisms. Also many large accumulations of leukocytes in the midst of which groups of organisms, two to six in a group, occur. Exudate is relatively scanty.

Stained smear.—Similar to 12. Mononuclear cells seem to be relatively more numerous than in last few specimens.

April 12, 1909. 11:30 A.M. Slide 14: (Pig seems somewhat asthenic. Tissues are swollen at abdominal wound. Wound is closed but bleeds readily. Small, pin-head nodules are palpable in right testicle. Left testicle is hardened; nodules similar to those in the right testicle are found.) Exudate is very scanty. One small drop obtained which shows very marked concentration of leukocytes about organisms, giving an appearance very similar to typical giant cells of the foreign body type.

Stained smear.—Mononuclear cells only, big, endothelial-like cells for the most part. They occur in huge masses, surrounding numerous organisms. All leukocytes in these masses seem to be losing their staining power; cytoplasm and nucleus is vacuolated.

This specimen was the last that could be obtained. Slide 14 was allowed to stand at room temperature. Examined the next day, it was found that long hyphae had grown out from many of the organisms which were inclosed in giant cells.

The guinea-pig was etherized two days after the making of slide 14. At autopsy the peritoneum, visceral and parietal, was dotted with grayish nodules from the size of minute points to nodules having a diameter of from 5 to 7 mm. The omentum was practically a mass of such nodules. The testicular surfaces also presented numerous grayish nodules, the largest being about 1 mm. in breadth. There was no excess of fluid in the peritoneal cavity. The nodules at first sight seemed to be retroperitoneal. Attempts to remove them showed that most could be peeled off without a great deal of difficulty, leaving a roughened opaque surface, while a few were considerably more adherent and, when removed, left a roughened opaque surface on which could be seen an occasional bleeding point. On cutting the nodules, some, especially the larger ones, were found to have soft centers; smears of this material showed polymorphonuclear leukocytes and oidiomycetes. Microscopically the nodules fixed in Zenker's fluid, imbedded in celloidin and stained with hematoxylin and eosin, were found to consist of masses of fibrin and leukocytes. The leukocytes were mostly of the large mononuclear, endothelial-like type, although in some isolated spots polymorphonuclear leukocytes predominated. Oidiomycetes were present in large numbers, usually in the center

of the nodule, and inclosed usually within cells having from 1 to 20 oval, well stained, peripherally placed nuclei. As a rule the nuclei numbered from 3 to 8.

The abundance of the cytoplasm of such cells was in direct proportion to the number of nuclei and took a marked uniform eosin stain with suggestion of a very finely granular structure. Sometimes the organisms were apparently free in the nodule; in such cases they formed the center of a small, circular, sharply circumscribed accumulation of polymorphonuclear leukocytes. There were no evidences of necrosis. The oidiomycetes themselves presented a variety of appearances. The budding forms frequently occupied the center of the small abscesses just mentioned; they presented a brightly shining, unstained, double-contoured external membrane, within which was an irregularly distributed, blue-stained, somewhat granular material which seemed, however, to form a lining layer just within the limiting membrane and not to be diffused to any great extent throughout the cell. In other organisms, the double-contoured membrane was unchanged and the material within the cell still took a blue stain; the latter, however, had seemingly shrunk away from the outer wall at all but one point, leaving a clear, unstained, new-moon shaped space. In still other organisms, this material had seemingly shrunk still more and was beginning to take an eosin stain; in others, it was beginning to lose its granular structure and to assume a homogeneous appearance, taking a sharp eosin stain. In such organisms as this, the central portion had sometimes become completely separated from the cell membrane so that the new-moon shaped space had grown into a complete circle; in other organisms, an equally pink-stained, homogeneous central portion was still in contact with the external membrane at one point, leading one to imagine that possibly the complete separation just described might be more apparent than real owing to the plane in which the organism had been cut.

Quite frequently no sign of "shrinking" was observed, the cells being a homogeneous pink without other modification. In those organisms which took the blue stain, it appeared as though the unstained portions within the capsule represented the homo-

geneous spherules described in the fresh specimens while the stained parts represented what might be termed the interspherular substance. A somewhat similar series of changes occurring in yeast *intra vitam* has been described by Potron.[17]

The peritoneum extended intact beneath most of the nodules. The cells of the peritoneum, however, had assumed a marked cuboidal or rounded shape and seemed to have broken contact with each other. In a few instances a typical organization was proceeding from the sub-peritoneal tissues into the overlying oidiomycetes containing exudate.

The kidneys were normal, as were the other organs. It will be remembered that some of the organisms in the peritoneal exudate of slide 14 grew. Oidiomycetes developed in pure culture from material taken from nodules at autopsy.

It does not appear to be necessary to report further experiments in detail. We shall summarize the course of events in the peritoneal cavity of a guinea-pig following the injection of an emulsion of living oidiomycetes in 0.85 per cent NaCl solution, basing our summary on numerous experiments such as the foregoing, on the autopsy findings of pigs dying or killed during the course of an experimental infection, and on observations made at exploratory operations, as follows: There is first a great transudation of fluid poor in fibrinogen and leukocytes into the peritoneal cavity. The few leukocytes present are mostly large mononuclears, though lymphocytes and polymorphonuclear leukocytes occur. After one or two hours the leukocytes begin to accumulate rapidly, and at the end of three or four hours, the peritoneal fluid, at first thin and watery, becomes very cloudy and quite viscid. This cloudiness and viscidity gradually increase as the fluid becomes more and more scanty, until finally, after three or four days, no more fluid can be obtained. If one opens the abdominal cavity of a guinea-pig 24 hours after the injection of a large dose of oidiomycetes, he finds an acute, diffuse, suppurative, and fibrinous peritonitis. The peritoneum is covered with a thick layer of turbid, sticky fluid in which are occasional yellowish-gray clumps. These clumps are soft and may be easily lifted from the peritoneal surface; in fact, they can hardly be said to be adherent.

The microscopical appearance of this exudate has been described above. The clumps consist of accumulations of phagocytic cells and groups of cells—the "rosettes" of Skchiwan—supported by a scanty fibrinous meshwork. After four days very little fluid will be found; the peritoneum may have a dull grayish tinge or may be about normal in appearance; it surely, however, will present numerous raised, convex, grayish nodules 0.5 to 5 mm. in diameter and sometimes whitish patches as much as 1 cm. in greatest extent, but these latter are not usual. As a rule, nodules and patches are easily detachable, leaving a dull, rough surface which sometimes may be slightly granular. They consist of masses of single large mononuclear leukocytes, some containing organisms, of polymorphonuclear leukocytes and masses of the phagocytic "rosettes," in a fibrinous meshwork. As the observations are extended to include lesions at longer and longer intervals following the injection, it is found that the nodules, beginning about the fifth day, become more and more difficult to remove, and, when removed, become more and more prone to leave a bleeding surface. Some seem to sink down into the tissue upon which they at first rested; the peritoneum grows over them; they become less and less prominent, and finally disappear entirely in the course of 15 to 30 days. Other nodules—and this seems to be especially true of nodules in the testicles following the intraperitoneal injection of 0.1 gm. of organism or more—commonly grow progressively larger for from 4 to 10 days, become somewhat soft and doughy, and then, in favorable cases, gradually harden, decrease in size and disappear entirely within 40 days. In unfavorable cases the process of enlargement and softening continues until, as in guinea-pig 75 (see p. 201), the scrotum becomes greatly distended, the overlying skin becomes involved, and the lesion finds an external opening. Microscopically, in the first type, blood-vessels are observed to grow into the "rosette" masses from underlying tissues, connective tissue is laid down, the nodules become young connective-tissue growths, and presently, the "rosettes" formed from free, phagocytic macrophages are transformed into morphologically typical Langhans giant cells of inflammatory granulation tissue merely by this replacement of their supporting fibrinous reticulum

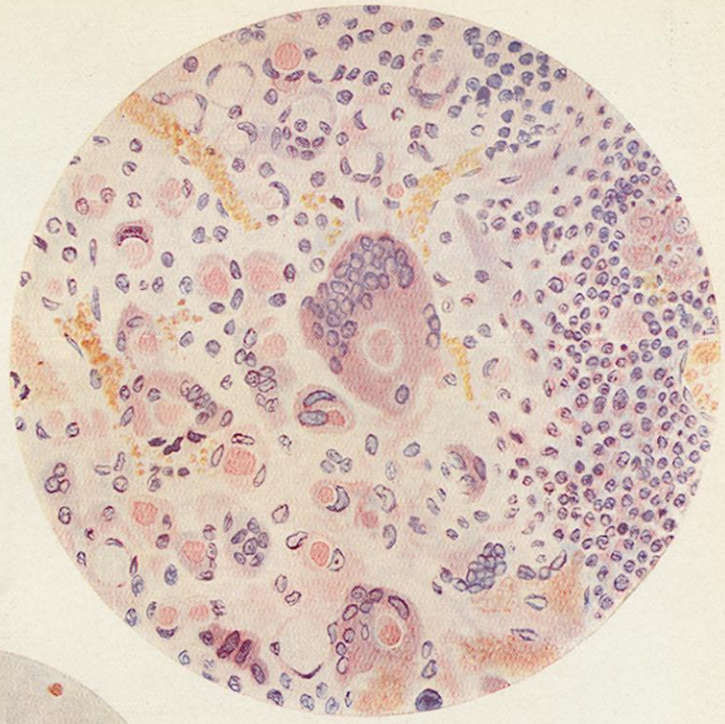
by a mesh-work of capillaries and their accompanying connective-tissue cells (see Fig. 5).

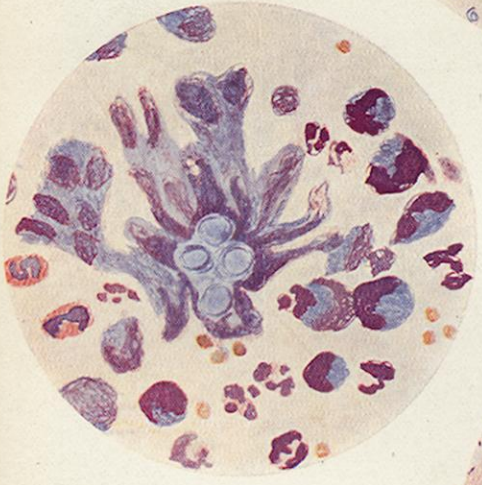
In the case of very large nodules such as those which formed in the peritoneal cavities of guinea-pigs 13, 37, 38, and 56 (see p. 204) the connective tissue growth may be so extensive as to cut off its own blood supply, thick-walled cysts being formed which contain a pus-like fluid. This material is not pus, however, but consists of cellular detritus, and, in the earlier stage, contains oidiomycetes showing various degrees of degeneration. In stained sections of this necrotic material from guinea-pig 13, autopsied 100 days after the last injection, one finds oidiomycetes which are practically normal in appearance, others have assumed appearances similar to those described in the nodules of guinea-pig 86, while others have gone still farther in that the cell membrane has either become thickened, homogeneous, eosin-stained, irregular in outline, or in other instances has disappeared leaving merely the inner homogeneous, eosin-stained spheres. Microscopical examination of the unstained detritus reveals bodies very similar to organisms in appearance but which are soluble in ether. In specimens from other nodules of similar age, guinea-pig 56, for instance, autopsied 116 days after last injection, the ether-soluble bodies are the only things which occur that in any way resemble oidiomycetes. Whether or not they bear any direct relation to the organisms has not been determined.

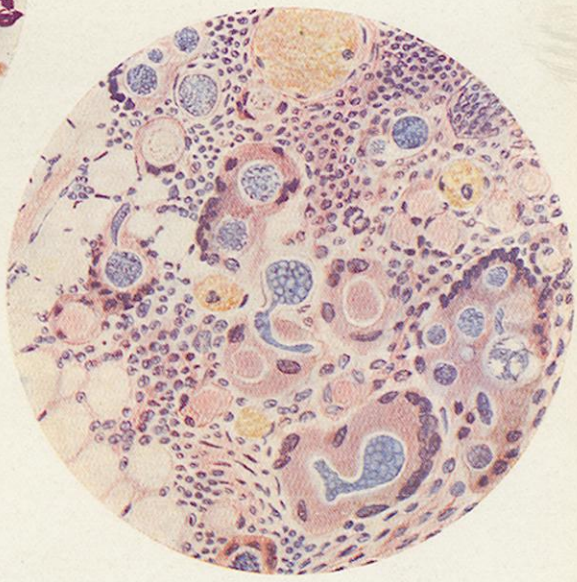
In the case of those nodules which become progressively larger and tend to soften, the bulk of the leukocytes consists of polymorphonuclears. In such masses, organisms commonly occur free; frequently they remain inclosed in Langhans giant cells which thus appear in stained sections as eosin-stained islets in the mass of blue-lobed nuclei. Such nodules occur to some extent in the early stages of all cases, but in the later stages of fatal cases only.

Mention has been made of nodules "in the testicles." This statement is not strictly correct since most nodules palpated "in the testicles" are merely in the peritoneal and fibrous tunics. Even in the most advanced cases the testicle itself may be and

PLATE 5.







generally is unharmed, as in guinea-pig 75 (see p. 201) in which at autopsy the testicles were found normal.

Besides the peritoneal lesions, macroscopic nodules have been found in the liver and in the lungs. In the liver they appear as white to grayish red patches 1–5 mm. in cross-section, rather sharply demarkated. In the lungs they appear as translucent droplets 0.5–2 mm. in diameter, occurring both sub-pleurally and on the cut surface. The animals seem to have died before larger nodules had time to develop.

Under the microscope in sections stained with hematoxylin and eosin, typical liver nodules appear as follows:

Section of the liver of guinea-pig 53 (for history, see p. 206; see also Plate 5). Slight passive congestion; slight central fatty infiltration; there is one area with a diameter of 4–6 liver lobules in which the parenchyma cells seem to have been replaced by widely dilated, erythrocyte-filled sinuses, and oidiomycetes inclosed in cells having from 1 to 20 nuclei. The multinucleated cells resemble closely foreign-body giant cells, excepting that the nuclei tend to be more rounded than those commonly seen in the latter cells, and, when oval in shape, their long axis tends to run parallel with the surface of the cell rather than centralward. This area is sharply marked off from the liver tissue by a narrow layer of parallel, wavy, pink-stained fibers, peripheral to which is quite a marked infiltration of mononuclear cells, mostly of the lymphoid type, though many are cells with abundant cytoplasm. The neighboring parenchyma cells are normal in most cases, although occasional islets of liver cells in which are found all stages of degeneration, even to that of complete necrosis, appear in the border tissue.

In the lungs, the nodules occur principally in the neighborhood of the bronchi or larger blood-vessels. They consist of dense accumulations of round and epithelioid cells with an organism at rare intervals; no necrosis; no edema; no exudation into alveoli. Aside from the nodules the lungs are normal.

PHAGOCYTOSIS IN VITRO.

The leukocytes.—Guinea-pigs were given intraperitoneal injections of a thick suspension of sterile aleuronat in 0.85 per cent NaCl solution. From examination of the exudate at hourly intervals it was found that, after a lapse of from 12 to 15 hours, the exudate was very rich in leukocytes and was still sufficient in quantity to be easily obtained. Of the leukocytes, from 25–50

per cent were large mononuclears, the remainder being mostly polymorphonuclear neutrophiles and eosinophiles. Both the fresh exudate and "washed" leukocytes were used. The "washed" leukocytes were obtained by washing the fresh exudate first in sodium citrate solution and then in one or two changes of physiological salt solution according to the ordinary opsonic technic. The suspension was then made up to the original volume of the exudate by the addition of 0.85 per cent NaCl solution. The fresh exudate clotted readily, so that, in using it, rapid work was necessary.

Exudate serum.—This was obtained by allowing the fresh exudate to clot spontaneously in test-tubes, centrifuging immediately, and removing the serum by means of a glass pipette.

The oidiomycetes were placed in a bottle of normal salt solution and shaken vigorously in a shaking machine for several hours before being used, in order to break up any clumps which might be present. An emulsion of the organisms in salt solution was then made which contained approximately 5,000 organisms per c.mm. In order to do this, counts were made of the organisms in the emulsion in the shaking bottle by means of the Thoma-Zeiss hemocytometer, and this emulsion was then diluted to the volume required as shown by the excess of organisms over the 5,000 per c.mm. which was desired.

Method.—The observations were made on hanging-drop preparations. The various materials were mixed on clean cover-glasses in triplicate, in the dilutions called for by the protocol, mounted on hollow ground slides, and the cover-slip sealed to the slides with paraffin. The preparations were incubated at 37° C. and observed after 30 minutes, after 3 hours, and after 24 hours. No stain was employed.

The first attempt was directed to determining (1) whether or not phagocytosis would occur *in vitro*, using (*a*) the fresh exudate and (*b*) washed leukocytes, and (2) the possible influence of guinea-pig serum upon the process.

Several preliminary experiments of which the following is a typical example were performed:

TABLE 6.
EXPERIMENT IN PHAGOCYTOSIS.

Slide No.	Organisms (Units of Emulsion)	Fresh Exudate (Units)	Washed Leukocytes (Units)	Normal Blood Serum (Units)	Exudate Serum (Units)	NaCl (0.85 per cent) (Units)
0.....	1	1	0	20	0	0
1.....	1	1	0	10	0	0
2.....	1	0	1	0	0	1
3.....	1	1	0	8	0	2
4.....	1	0	1	0	1	0
5.....	1	1	0	6	0	4
6.....	1	0	1	1	0	0
7.....	1	1	0	4	0	6
8.....	1	1	0	2	0	8
9.....	1	1	0	0	0	10
10.....	1	1	0	0	0	0

The final examination of these slides after 24 hours at 37° C. disclosed the following conditions:

Slide 0: Well mixed; many red cells; leukocytes occur singly but also very frequently in groups of two to a dozen or more—most commonly three or four cells per group. Organisms may or may not, infrequently not, be found within these groups. The rule appears to be for the leukocytes to pay no attention to the oidiomycetes. Very rarely a single leukocyte may be seen which has partially or totally engulfed an organism.

Slide 1: Organisms occurring singly are seen only within leukocytes. Organisms occurring in clumps are always surrounded and, to be perceived by careful focusing, engulfed by leukocytes. Leukocytic clumps are found in the absence of organisms, but not to the extent observed in slides 2, 4, and 6 (see below). Many large leukocytes with abundant cytoplasm occur; they are the actively phagocytic cells. There are many red blood corpuscles, but very few of them have been ingested.

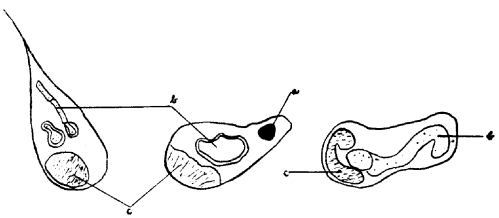


FIG. 1.—Types of phagocytic cells: *a*, erythrocytes; *b*, organisms; *c*, cell nucleus.

Slide 2: Leukocytes and organisms are quite numerous. Both types of cells, leukocytes and oidiomycetes, occur singly, by twos and threes, and in large groups, the organisms to a greater extent than the leukocytes. The cells are well intermixed and frequently approximate each other, but this is not marked enough to suggest phagocytosis or even positive chemiotaxis.

Slide 3: Very similar to slide 1, excepting that there is more extensive ingestion of red cells. In some cases leukocytes are merely grouped about organisms, in others there is not only grouping, but phagocytosis.

Slide 4: Similar to slide 2, excepting that two leukocytes are present which appear to be phagocytic. Each has partly surrounded an elongated blastomycete.

Slide 5: Many red blood cells are present. A good many of them have been phagocyted. Organisms are present in fair numbers and occur for the most part

within single, or plasmodial masses of, leukocytes. It is noticeable that the leukocytes which take up the red blood corpuscles also take up the organisms. It is common to find a leukocyte which contains 2, 3, or even 4 red cells in addition to an organism. Such large cells seem to be less numerous here than in slide 3. They assume bizarre shapes—greatly elongated, spindle forms, star-forms, and various other irregular outlines, with long, slender projections. This is especially noticeable about the edges where the drop is thin.

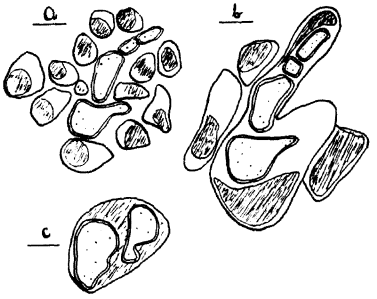


FIG. 2.—Sketches of two strikingly similar oidiomycetic groups to illustrate the difference between (*a*) mere grouping of leukocytes, and (*b*) grouping with phagocytosis; (*c*) shows a leukocyte containing two organisms. These sketches also illustrate the type of cell which is actively phagocytic.

Slide 6: Same general appearance as slide 4.

Slide 7: Fewer cells of all kinds: otherwise similar to slide 5.

Slide 8: Cells of all kinds seem to be still fewer than in slide 7. Absolutely free organisms are quire rare; on the other hand, ingestion is not always complete and many of the groupings suggest that shown in Fig. 2. This appears to be due in some degree to a dearth of the big leukocytes, since where they occur ingestion is as complete, as a rule (there are exceptions), as their bulk makes possible, as is suggested in the accompanying sketch.

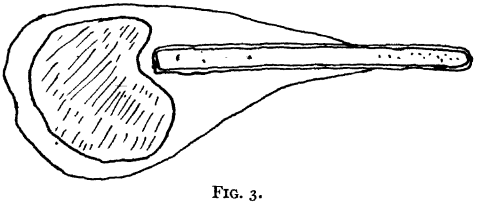


FIG. 3.

Red blood corpuscles have been phagocyted quite freely but by no means to the degree, relatively, that is evident in the case of the oidiomycetes.

Slide 9: Complete ingestion of single and budding organisms occurs with fair frequency; on the other hand, in

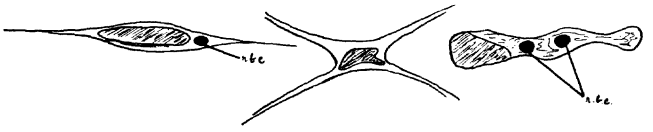


FIG. 4.

FIG. 5.

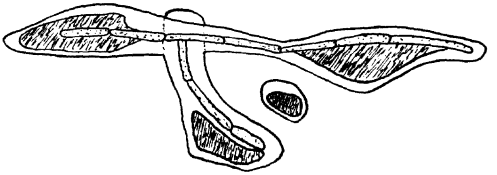


FIG. 6.

many instances the leukocytes seem to be entirely indifferent to the oidia. In plasmodial masses which are in relation to organisms there is generally more or less com-

plete phagocytosis; contrarywise, places are not hard to find where mere grouping, with little or no phagocytosis, occurs.

Slide 10: Conditions here are very similar to those in slide 0. If anything, phagocytosis may be slightly less marked, something of a hair-spitting distinction, however.

From this experiment it appeared (1) that the leukocytes of the fresh exudate, either without the addition of normal serum or when mixed with excessive quantities of normal serum, do not phagocyte oidiomycetes as well as such leukocytes plus moderate quantities of serum; (2) that washed leukocytes do not phagocyte oidiomycetes readily, when suspended in simple salt solution, when suspended in salt solution plus normal serum, or salt solution plus exudate serum; (3) that phagocytosis is carried on in the fresh exudate *in vitro* by cells of the same type as those which are responsible for the major part of the work in the peritoneal cavity.

An extensive series of further experiments was planned with the idea of testing thoroughly the above deductions. Unfortunately, the adoption of the mycelial mode of growth by the organism interrupted the work while it was still far from being complete, so that the results reported below must be regarded as purely tentative.

The summary of events follows: (1) It appears that the leukocytes of the undiluted fresh exudate are very actively phagocytic; complete phagocytosis is the rule within 30 minutes at 37° C.; that is, as a rule, every organism in the preparation will have been ingested within that space of time. The leukocytes commonly form a plasmodial mass about the oidiomycetes, the mass being surrounded by a narrow clear zone of fairly uniform width which separates it from the surrounding leukocytes. If the exudate is diluted by salt solution, normal serum, immune serum or exudate serum, the degree of phagocytosis seems to decline, roughly, with the increase of the dilution. In other words, the more widely the organisms are separated from the leukocytes, the less apt is phagocytosis to occur. The organisms do not seem to be strongly chemiotactic in the sense that leukocytes will be attracted to them from a distance, the ingestion of the oidiomycetes by the leukocytes seems to be a sort of contact phagocytosis. The sera

do not encourage phagocytosis to any greater extent than does physiological salt solution.

(2) As was indicated in the earlier work, washed leukocytes are very inconstant with respect to their ability to phagocyte oidiomycetes. The addition of the various sera mentioned above does not help matters. It was thought that perhaps the manipulation to which they were subjected injured the leukocytes in some way and an effort was made to obviate this as much as possible by decreasing the number of washings and the length of time in the centrifuge, by keeping the solutions at 37° C., etc., but without effect. Once in a while such leukocytes ingested organisms, but, in so far as could be determined, with neither rhyme nor reason. As these leukocytes were capable of ingesting carmine granules as well as an occasional organism it appeared that they had not lost their powers entirely; why, then, this failure to engulf oidiomycetes as actively as the leukocytes in the fresh exudate? Several explanations were suggested. The technic may have been unsuited to the materials with which the work was being done. Savtchenko[14] found that the mononuclear cells of the peritoneal exudate of guinea-pigs have a tendency to clump and not to phagocyte after centrifugation in citrate solution. Brisco[24] has made a similar observation with respect to the alveolar cells of the lung. It was noted that the ingested carmine granules were much smaller than the oidiomycetes. Possibly the leukocytes had been injured sufficiently to deprive them of the ability to envelop such giants as the yeasts cells but still retained vitality enough to engulf smaller particles. Again, the fresh exudate was quite viscid and fibrin threads formed within it in a very short time after the mixtures were made. Possibly the viscidity of the medium and the fibrin network were the factors which enabled the leukocytes to extend themselves over the organisms. It would be interesting, in this connection, to observe the effects of the addition of a solution of gelatin upon the phagocytic activity of the washed leukocytes. This might also be the key to the effects of dilution on phagocytosis in the fresh exudate.

(3) The proposition that phagocytosis is carried on in the fresh exudate *in vitro* by cells of the same type as those which are respon-

sible for the major part of the work in the peritoneal cavity is borne out by further observations. This does not mean that, either within the peritoneal cavity, or out of it, all the work is done by the macrophages. Such is not the case; polymorphonuclear leukocytes frequently take part in the work, and sometimes are the only leukocytes present in the plasmodial masses. They, of course, are too small to ingest any but the smallest oidiomycetes. Nevertheless, they are active in surrounding and "hemming in" the organisms. On the other hand, there is no room for doubt that the bulk of the work is done by the macrophages.

In three experiments, normal and immune serum and sodium citrate solution seemed actually to inhibit phagocytosis in fresh exudate, while the leukocytes in the salt-solution dilution of the same exudate were actively phagocytic. No explanations of these results have suggested themselves.

Fresh normal rabbit serum was mixed with guinea-pig peritoneal exudate. The leukocytes in the exudate became spherical in shape, and were strongly agglutinated within 30 minutes at 37° C. The observations were not carried further. Needless to say, no phagocytosis occurred in such preparations.

These experiments seem to indicate that opsonins are, at least, not of great importance in the phagocytosis of the oidiomycetes used in this work by the leukocytes of the peritoneal exudates of normal and immune guinea-pigs.

SUMMARY AND CONCLUSIONS.

Oidiomycosis in the guinea-pig, following intraperitoneal inoculation, is characterized, in fatal cases in male animals, by a gradually developing cachexia accompanied as a rule by a steady loss of weight which is especially marked during the last three or four days of life and by the development, in pigs of 400 gm. or over, of palpable nodules in the testicles in from three to seven days after inoculation. In males weighing 400 gm. or less the nodules in the testicles are inconstant, and, when present, may be difficult to palpate owing to the fact that they occur frequently about the upper pole of the testicle and about the neck of the scrotal sac—points which may be difficult to feel in small animals. The appearance of palpable nodules in the skin or anterior abdomi-

nal wall has been noted but may be avoided in a great measure by rinsing the outside of the needle before making the injection. In female guinea-pigs symptoms are usually entirely lacking; they survive intraperitoneal doses which kill the males, a point which should be borne in mind when testing the pathogenicity of yeasts for guinea-pigs. The scrotal sac of the male pig seems to afford a *locus minoris resistentiae* to which the females have no counterpart. The reasons for the lower resistance of this particular corner of the peritoneal-lined cavity are not apparent. Postmortem examination of animals dying of the disease reveals multiple grayish nodules from 0.1 to 10 mm. in diameter on all peritoneal surfaces. All such nodules contain oidiomycetes, as may be demonstrated in microscopic sections and in cultures. Most nodules have softened centers, especially the large nodules which are found in the testicles, in which oidiomycetes and great numbers of polymorphonuclear leukocytes occur. The lungs and liver may also present small areas of cellular infiltration about oidiomycetes. There are no constant changes in the various organs.

Recovery from an infection is accompanied by a low grade of immunity which manifests itself in a somewhat more speedy recovery from subsequent infections, and by the development of a slight temperature for a few days immediately following reinoculations.

Repeated injections of an "extract" of oidiomycetes lead to the development of an immunity in guinea-pigs which is characterized mainly by the more rapid walling off of organisms injected into the peritoneal cavity, and by a more rapid disappearance of the lesions which appear in the testicles.

The sera of guinea-pigs immunized by repeated intraperitoneal injections of living organisms or of an extract of organisms develop precipitating substances against the "extract" in from 50 to 79 per cent of the cases, which may be manifest in a dilution as high as 1:20. Normal sera occasionally precipitate with the "extract" in lower dilutions. Substances which will agglutinate oidiomycetes suspended in dilutions of the sera are not formed. Specific amboceptors also do not appear to be developed. Opsonins have not been demonstrated. Prolonged exposure of oidiomycetes to immune or normal serum at 37° C. seems to impair the vitality of

the organisms—judging by their diminished power of growth when transferred to suitable media—as compared with that of similarly tested control tubes containing organisms suspended in physiological salt solution. The reaction of anaphylaxis may, under suitable conditions, be obtained with the "extract" in guinea-pigs. The "extract" is toxic; prolonged administration by the intraperitoneal route results in cachexia and death usually by secondary infection; judiciously used, it may have a favorable effect upon the course of an oidiomycetic infection.

Oidiomycetes injected into the peritoneal cavity of guinea-pigs are rapidly taken by leukocytes, macrophages principally, which form plasmodial masses and become attached to the peritoneal surface at first by fibrinous, later by fibrous, adhesions, the mass of cells grouped immediately about the organisms presently assuming the structure of typical Langhans giant cells. In cases ending in recovery, the organisms may grow for a time, but eventually degenerate and disappear; the nodule within which they were, being absorbed with them. In fatal cases the nodules increase in size and become soft; many of the organisms degenerate, but others multiply; the inclosing giant cell disintegrates and there ensues an infiltration of polymorphonuclear leukocytes *pari passu* with the enlargement and softening of the nodule. Sometimes the organisms are surrounded by polymorphonuclear cells from the beginning; such a mass may in turn be inclosed by layers of macrophages. Just what such a condition may mean as regards the prognosis of the lesion has not been determined.

The organisms do not tend to penetrate the peritoneal surface. It seems probable that they leave the peritoneal cavity, only in case there has been actual tearing of the lining tissues. Oidiomycetic nodules were described in the liver and lungs of a few animals. The belief seems to be justified that such generalization of the infection followed the introduction of the organisms into the circulation by mechanical means, because the condition occurred almost solely (there were two exceptions) in animals in whose peritoneum one could detect tears, as in the animals used for the study of phagocytosis *in vivo*.

The mode of defense of guinea-pigs against oidiomycetes in-

jected into the peritoneal cavity appears to consist, firstly, in phagocytosis and intracellular digestion; secondly, in a walling off and encapsulation of the phagocyted organisms by connective tissue; and, thirdly, upon a somewhat ill-defined, and possibly questionable, unfavorable influence of the serum upon the vitality of the organism. These agencies act but slowly; oidiomycetes may retain their power to grow on artificial media for days, and their characteristic staining properties for weeks, in the inflammatory nodules of supposedly immune pigs. Specific antibodies are but poorly developed.

The author is indebted to Dr. Howard T. Ricketts for advice and assistance, and for the use of unpublished experiments, portions of which have been incorporated in the body of this paper; to Dr. H. G. Wells for advice and assistance, and to Mr. William F. Peterson who has contributed generously of both time and energy to the promotion of the latter part of this work.

REFERENCES.

1. METSCHNIKOFF. *Virchow's Archiv*, 1884, 96, p. 177.
2. RIBBERT. *Der Untergang pathogener Schimmelpilze im Körper*, Bonn, 1887.
3. SANFELICE. *Centralbl. für Bakt.*, Abt. 1, Orig., 1896, 20, p. 219.
4. CHARRIN AND OSTROWSKY. *Comptes rendus Soc. de Biol.*, 1896, 48, p. 743.
5. ROGER. *Comptes rendus Soc. de Biol.*, 1896, 48, p. 728.
6. SCHATTENFROH. *Arch. für Hyg.*, 1896, 27, p. 234.
7. GILKINET. *Arch. de méd. expér.*, 1897, 9, p. 881.
8. JONA. *Centralbl. für Bakt.*, Abt. 1, Orig., 1897, 21, p. 147.
9. OBICI. *Zeiglers Beiträge*, 1898, 23, p. 197.
10. SKCHIWAN. *Ann. de l'Inst. Pasteur*, 1899, 13, p. 770.
11. RICKETTS. *Jour. Med. Res.*, 1901, 6, p. 377.
12. MALVOZ. *Ann. de la soc. méd.-chir. de Liège*, 1901, quoted from Sanfelice (15).
13. MALVOZ. *Centralbl. für Bakt.*, Abt. 1, Orig., 1901, 29, p. 668.
14. SAVTCHENKO. *Ann. de l'Inst. Pasteur*, 1902, 16, p. 106.
15. SANFELICE. *Centralbl. für Bakt.*, Abt. 1, Orig., 1902, 32, p. 360.
16. WLAEFF. *Compt. rendus Soc. de Biol.*, 1902, 54, p. 412.
17. POTRON. *Blastomycetes dans les tissues.* Thèse, Nancy, 1903.
18. FABOZZI. *Centralbl. für Bakt.*, Abt. 1, Ref., 1906, 39 (Abstract), p. 282.
19. SPIETHOFF. *Jahrbücher der Hamb. Staatskrankenanstalten*, 1903–4, 9, Heft II, p. 167.
20. BOWEN AND WOLBACH. *Jour. Med. Res.*, 1906, 15, p. 167.
21. CHRISTENSEN AND HEKTOEN. *Jour. Amer. Med. Assoc.*, 1906, 47, p. 247.
22. HAMBURGER. *Jour. Infect. Dis.*, 1907, 4, p. 201.
23. MARCO DEL PONT. *Bull. de l'Inst. Pasteur*, 1907, 5, p. 46.
24. BRISCO. *Jour. Path. and Bact.*, 1907, 12, p. 66.
25. BROWN. *Jour. Amer. Med. Assoc.*, 1907, 48, p. 743.
26. HEKTOEN. *Jour. Amer. Med. Assoc.*, 1907, 49, p. 1071.
27. MONTGOMERY AND ORMSBY. *Archives Int. Med.*, 1908, 2, p. 1.
28. ROTHE. *Deut. med. Wchnschr.*, 1910, 36, p. 30.
29. WIDAL, ABRAMI, JOLTRAIN, BRISSAUD, ET WEILL. *Ann. de l'Inst. Pasteur*, 1910, 24, p. 1.