THE GROWTH OF MYCOBACTERIUM PARATUBERCULOSIS IN TISSUE CULTURES

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THESIS

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Introduction

Since the discovery of the etiology of Johne's disease some 40 years ago by Johne and Frothingham, much has been added to the knowledge of this perplexing disease. But, in spite of researches conducted by many workers both in this country and abroad, comparatively little is known about the true nature of the organism and the resulting disease. The slowness of growth on laboratory media is a great handicap to the study of the biology of Mycobacterium paratuberculosis; likewise, the failure of all attempts to produce in the common laboratory animals any disease resembling in the least Johne's disease of cattle and the slowness with which the disease develops in bovines have greatly limited experimental work similar to that fruitful of such revealing results in other diseases of animals and humans.

Since Harrison (1) first succeeded in growing tissue in vitro in 1907, many workers have utilized this method of procedure for the study of the reaction to organisms of tissue grown in vitro. It has been to the experimenter what the roentgen rays were to the diagnostician, a means of peering into the vital processes of the body, a key to a hitherto unopened gateway, giving entrance to the secrets of vital cytologic processes. It is particularly fitting that tissue culture methods be applied to the study of the biology of Johne's bacillus, not only because of difficulties encountered in other methods but because of the slow rate of growth of the organism and its low toxicity for cells in vitro and in vivo.

The present work herein reported was inaugurated with the idea of growing, if possible, Johne's bacilli in vitro in cultures of animal tissue and of studying under these carefully controlled conditions, free from the general systemic influences of the body, the reaction of individual cells to the presence of the organism. It is realized that the report is incomplete and that the problem is worthy of more prolonged and detailed investigation, but the time allotted does not permit of such detail. Undoubtedly worthy results could have been obtained by studying cultures of longer growth, cultures inoculated after growth was well commenced, and particularly cultures of bovine tissue. It is hoped that a report on these studies can be made at a later date.

Review of Literature

It is generally accepted that the cultivation of tissue outside of the organism was successfully accomplished for the first time by Harrison (1) in 1907, working in the Anatomy Department of Johns Hopkins University. Previous to this Leo Loeb (2) stated that he had cultivated cells outside as well as inside of the body but did not publish his method. In 1902 he published a method of cultivating tissue inside of the body by placing fragments of the skin of guinea pig embryo in agar and coagulable serum and inserting them into adult guinea pigs (3). Harrison's method, published in 1907, consisted of placing embryonic tissue (i. e., central nerve tissue) of the frog in coagulable lymph. Growth of nerve

fibers was observed. Later Burrows (4), working under Harrison, improved the technique, substituting blood plasma for lymph, and adapted it to cultivation of chick embryonic tissue. Borrows and Carrel (5) then succeeded in growing adult mammalian tissue, and Carrel (6), by adding chick embryonic extract, obtained a permanent and uninterrupted multiplication of fibroblasts. Such strains of fibroblasts have been maintained for many years (7).

The changes undergone by lymphoid tissue when cultivated in vitro in medium composed of blood plasma and tissue extract are described by Maximow (8) in a series of articles, and it is shown that the transformation of the various cell types can be followed with great accuracy. "The cell types are: 1. Fibroblasts. They proliferate, grow radially as spindle-shaped cells into the plasma and form the framework of the new tissue zone, surrounding the explant. 2. Endothelium of blood vessels. It partly produces new capillary sprouts, partly becomes transformed into fibroblasts. 3. Reticular cells. They are mobilized as large, proliferating, ameboid and phagocytic, sometimes epithelioid polyblasts or macrophages, storing dyes, forming, in the presence of foreign bodies, through fusion, multinucleated giant cells, and sometimes accumulating in large numbers in the liquid on the surface of the explant. They are easily distinguished by their pale, irregular nucleus, their ameboid protoplasm and the typical pigment granules, staining green with eosin-azure. 4. Lymphocytes. A part of them migrate into the surrounding medium and them degenerate; a part remain in the explant and

in the loose new tissue zone and here continue to proliferate and transform themselves into plasma cells, into ameboid phagocytic polyblasts or into granulocytes."

Although tissue culture methods were utilized by Carrel and others for the study of cell physiology, pathologists were slow to grasp the opportunity offered to study action of organisms upon isolated cells. In 1920 Lewis (9) observed that vacuoles were formed in the cells of tissue cultures as the result of inoculation with Eberthella typhi. Previously Smyth (10) studied the action of several forms of organisms upon the cells of chick embryos in vitro, among which was the tubercle bacillus. He observed a clustering of small round cells interpreted as lymphocytes around colonies of the bacilli after the third day of growth with the bacilli. He reported phagocytosis of the organisms by connective tissue and epithelial cells. Giant cell formation was observed. Smith, Willis, and M. R. Lewis (11) confirmed some of the findings of Smyth. Their results were not identical for they substituted the artificial Lewis medium for blood plasma medium and cultivated chick embryonic tissue inoculated with avian tubercle bacilli. They could not determine any growth of tubercle bacilli in the medium used. Phagocytosis was very slight, and only accidental engulfing of single bacilli was observed. They emphasized the fact that the cells were not attracted by the bacilli in any degree. No change induced by the presence of the bacilli in the cultures could be discerned.

A most excellent work on the reaction of tissue of the rabbit in vitro to tubercle bacilli was reported by Maximow

(12). Lymphoid tissue from the mesenteric lymph nodes, tissue from the omentum, and the common connective tissue were grown and inoculated with tubercle bacilli. Two strains of human type were used. One was recently isolated from sputum and highly virulent; another was of very low virulence and grew well on artificial medium. He observed that the tubercle bacilli grew well in the cultures and were actively engulfed by certain cells. The cultures were generally able to overcome the virulent strain so that in seven to eight days after inoculation only traces of them could be discovered in the cultures, but not so the avirulent bacilli. They multiplied freely and completely overgrew the cultures after three weeks of life in vitro. He emphasized the fact that, even in those cultures containing great quantities of tubercle bacilli, the cells of the tissue did not perish but displayed a series of reactive changes which duplicated closely the tuberculous process as it is found in the organism. This is in marked contrast to the behavior of the cells to other microorganisms for in cases where various ones were inoculated into cultures (Smyth, Lewis) the cells and the bacteria could not be kept alive together for any appreciable length of time. Likewise, when tissue cultures become accidentally contaminated with saprophytic organisms, the cells are absolutely unable to resist the intruders and are soon killed.

Maximow concluded that the chief role in the cultures of lymphoid tissue was played by the reticular cells. They were usually mobilized and formed active wandering cells, polyblasts (macrophages), and migrated into the surrounding medium

and readily engulfed the tubercle bacilli wherever they encountered them. Those reticular cells which were converted into polyblasts in lymphoid tissue and remained in the explant also engulfed the bacilli which penetrated everywhere between the cells. Many reticular cells engulfed bacilli while still maintaining their fixed position with reference to the reticulum. Epithelioid and giant cells were formed as a result of phagocytosis of bacilli and also by cells not in contact with organisms. This latter reaction was concluded to be the result of diffusible toxins. Mitosis and a peculiar hypertrophy of reticular cells were observed to be direct results of the chemical stimulation in inoculated cultures.

In inoculated cultures of lymphoid tissue, lymphocytes showed a remarkable disposition to clump together, not observed in controls. Some of them migrated into the medium and perished; others transformed themselves into polyblasts which behaved exactly as those of reticular origin and could not be distinguished from the latter except in cases where those of reticular origin retained a typical greenish pigment after the eosin-azure stain.

A golden yellow pigment was observed in epithelioid and giant cells as a residue from the intracellular digestion of tubercle bacilli. Maximow further states that the intracellular digestion of bacilli was continued and probably highly increased within the giant cell. In other cases bacilli were not destroyed within the epithelioid cells, but multiplied rapidly. Such cells full of bacilli were observed to retain their vitality for a long time, move about as usual,

and even divide mitotically. Bacilli did not multiply so rapidly within the giant cells for the digestive powers of the latter were probably far greater than those of the macrophage. The principle mode of formation of giant cells was by fusion of mononuclear cells. This phenomenon was looked upon as a further step in the process of the clustering of cells previously mentioned. In a few instances giant cells with constricted and fragmented nuclei were observed and interpreted as being the results of amitotic division. They were not of common occurrence. In some cases the formation of giant cells was believed to be due to the direct fusion and hypertrophy of lymphocytes. In these instances the polyblastic stage was omitted.

Maximow made no reference to any important part played by granulocytes in the tuberculous process in vitro. He mentioned that they are of common occurrence in the cultures, but as the tuberculous picture develops they mostly degenerate, apparently being sensitive to the presence of the bacilli. The greater number of his cultures were not inoculated until after four or five days growth in vitro after the granulocytes had lost much of their initial activity.

A few tubercle bacilli were seen to enter the protoplasm of lymphocytes in a passive way. Later when they were transformed into epithelioid cells, active phagocytosis took place.

The literature on Johne's disease is much less voluminous than that on tuberculosis. Its slowly progressive and nonspectacular features have curbed the interest of all but a few workers in this country (Hallman and Witter, 16). No attempt

will be made to review completely the results of researches on Johne's disease nor to describe in detail the resulting symptoms and lesions. For a very comprehensive bibliography and review the reader is referred to the excellent bulletin of Hastings, Beach, and Mansfield, of the Wisconsin Experiment Station (13). Also Hallman and Witter have recently reported the results of a detailed study of the pathology of Johne's disease.

The disease is characterized by a patchy and diffuse thickening of the intestinal wall and involvement of the mesenteric lymph nodes. In the initial stages the intestinal lesions of Johne's disease have a histology identical with that of tuberculosis (13). Later the necrosis and caseation, as well as fibrosis and resulting focal nature of tuberculosis, are absent. Extreme infections give a picture of an intestinal mucosa diffusely thickened by enormous numbers of macrophages and a variable number of giant cells which in many cases contain great quantities of acid-fast rods. There is apparently a total absence of any fibrotic or degenerative changes.

In a recent publication Theobald Smith (14) states that in his experience epithelioid cells always contain acid-fast rods and concludes that no diffusible toxin is formed, but the resulting formation of epithelioid cells is the direct result of phagocytosis of organisms. He also observes, "Any association of macrophages with the local formation or disintegration of tubercles or in the expanding proliferation of epithelioid cells in paratuberculosis was not observed at any time." This former observation is in direct contradiction to

that of Hallman and Witter, who found early cases in which, although typical collections of macrophages and giant cells were fairly numerous, acid-fast rods were relatively infrequent. Smith also made the observation that there is an engulfing of erythrocytes by phagocytes in the spleen.

Technique

Glassware. Pyrex glassware was used wherever feasible. It was thoroughly scrubbed with a washing powder, rinsed, and placed in an acid cleaning solution for 24 hours to remove excess alkali. It was then washed in running water for two hours and in two changes of distilled water. Pipettes were dried in alcohol and then in ether and wrapped in paper or inclosed in large glass tubes or copper containers. All glassware was sterilized in the hot air oven at a temperature not exceeding 140° C. for one hour.

Tyrode's solution. This solution was made up in a sterile, two litre Pyrex volumetric flask. The water was triple distilled in Pyrex and autoclaved immediately before using. All chemicals were of the purest obtainable. The composition is as follows:

NaCl	8.000 grams
KCl	0.200 "
$CaCl_2$ anhydrous	0.200 "
$MgCl_2 \cdot 6H_2O$	0.100 "
$NaH_2PO_4 \cdot H_2O$	0.050 "
$NaHCO_3$	1.000 "

Glucose	1.000 grams
H_2O	1 litre

The ingredients were added to about one-half of the water in the order given, and then the remainder of the water was added. It is recommended that the solution be filtered through a Berkefeld filter with a minimum of metal fittings although a Zeitz filter was used with good success in this work. The sterile solution was stored in Pyrex test tubes in the refrigerator until used.

Plasma. Both chicken and guinea pig plasma were used. The chicken plasma gave better results and resisted liquefaction by cells better than that of the guinea pig. Heparin was added to the blood immediately after drawing at the rate of 1 mg to 20 cc. for chicken blood. Slightly more than this was used for guinea pig blood. The blood was drawn under sterile precautions from the heart and placed in an iced, paraffined tube which contained the heparin solution (10 mg. of heparin per 1 cc. of .85% NaCl). It was then centrifuged at high speed for five minutes in cooled centrifuge buckets. The plasma was pipetted off and stored in the refrigerator in paraffined tubes.

Embryonic extract. Embryonic extract was obtained from nine day chick embryos. The eggs were opened by carefully cracking the shell at the large end, being careful not to break the outer membrane. The shell was carefully lifted off with small forceps and the outer membrane cut away with small, sterile scissors. The inner membrane was then cut away in the same manner, exposing the embryo, which was lifted out

and placed in a large, short test tube and minced into fine pieces with scissors. A Latapie tissue crusher would be useful here, but none was available. Two parts of Tyrode's solution were then added to one of minced embryo. This mixture was frozen until solid in salted, cracked ice to destroy the embryonic cells. If this precaution is neglected, the chick cells are likely to get into the culture, where they grow with great vigor. After freezing, the extract was placed in the 37°C. incubator for one hour, centrifuged, and the supernatent fluid pipetted off. This was diluted with an equal part of Tyrode's solution before using.

Serum. This consisted of 0.75 cc. of 30 per cent guinea pig serum in Tyrode's solution and 0.05 cc. of a 0.1 per cent heparin solution. This mixture composed the fluid phase of the medium. The heparin has a slightly inhibitory effect on the cell growth and effectively prevents liquefaction of the medium by a too rapid growth of cells and consequent accumulation of metabolites (16). In some instances after the first transfer the heparin was omitted from the fluid phase.

All material for medium was stored in the refrigerator in a closed jar containing about five per cent CO_2. This helped to maintain the pH at the proper level. All materials were cultured in veal infusion broth before using.

Bacterial cultures. Two strains of Johne's bacilli were used in this study. Both had been grown on laboratory media for some time. They grew very slowly, several months elapsing before the colonies reached appreciable size. Both refused to grow on medium containing no Mycobacterium phlei. Each

strain had previously been used for the production of Johnin.

Inoculation of the tissue cultures was made by emulsifying a loopful of the growth from solid medium in 2 cc. of Tyrode's solution and placing a small drop of this emulsion into each culture. An equal number of cultures was left uninoculated for controls. This method gave a rather heavy inoculation and distributed the organisms throughout the culture medium and made possible a study of organisms both in contact with growing cells and those at some distance from the explant.

Tissue cultures. The cultures were made upon a round coverslip in a solid plasma clot after the method of Maximow (8). The medium consisted of equal parts of diluted plasma (one part of plasma to two parts of Tyrode's solution), Tyrode's solution, serum, and embryonic extract.

All operations were carried on in a closed culture room in which the bench had been thoroughly washed down fifteen minutes previously and the room closed tightly until operations were commenced. The guinea pig from which the tissue was obtained was anesthetized, the abdomen shaved, and the skin surface cleansed and disinfected first with 70 per cent alcohol and then with tincture of iodine. The tissue was removed with sterile instruments and place in warm Tyrode's solution and taken immediately to the culture room. It was then cut into small cubes, less than 1 mm. square, with two very sharp knives, taking care that at no time it be exposed to dessication.

Large coverglasses, 50 x 44 mm., were then placed upon sterile, black tiles and covered with one half of a sterile

petri dish. A drop of Tyrode's solution was placed in the center of each slip and a round coverslip lightly placed on this drop. The coverslip adhered tightly if the drop was not too large, and the tissue was placed on this and immediately covered with one or two drops of the medium mixture. This clotted in a short time, after which a drop of the fluid phase was placed on the culture. The coverglass was immediately inverted over a large, hollow glass slide which had a 37 mm. concavity. Previously a drop of sterile, melted petrolatum was placed at each corner to make it adhere to the slide. The preparation thus made was sealed with melted paraffin to prevent evaporation of fluid and placed in the incubator. It is important that all operations be carried on as speedily as possible and that the tissue at no time be exposed to drying.

The cultures were transferred every fourth or fifth day by opening the preparation, removing the round coverslip and culture, washing in Tyrode's solution for a period of 20 minutes, returning to a clean, large coverslip, placing a fresh drop of the fluid medium on the culture, and again inverting over a hollow slide.

Guinea pig spleen was selected as the tissue to be used, not because it was considered more preferable than bovine lymphoid tissue but because the latter was not easily obtainable. However, it had the advantage of being from an animal considered resistant to Johne's disease, which allowed a study of the mechanism of such resistance.

All cultures were incubated at a temperature of 39° C.

They were observed twice daily under a microscope with a warm stage. In addition, one culture was kept under constant observation in a microscope incubator.

Cultures were fixed at various ages for 15 to 20 minutes in Maximow's modification of Zenker's solution, in which ten per cent neutral formal is substituted for five per cent glacial acetic acid. This was accomplished by removing the round coverslip bearing the culture and floating it face down upon the fixing fluid. It was next washed in frequent changes of distilled water for 24 hours and dehydrated in ascending strengths of alcohol followed by absolute alcohol for 24 hours. In the absolute alcohol the culture was removed from the coverslip by shaving off with a sharp razor blade which was wet with alcohol. It was then placed in cedar oil for four to six hours, infiltrated in an oven in paraffin having a melting point of 52° C., embedded in paraffin, and sectioned. Some of the cultures were fixed in ten per cent neutral formalin and stained in toto for blood pigments or fats.

Most of the cultures were sectioned serially on the microtome at a thickness of 7 u., stained in Ziehl-Neelsen carbolfuchsin at room temperature for 10 to 15 minutes, and decolorized for 30 seconds to one minute in 70 per cent alcohol containing one per cent HCl. This decolorized the surrounding tissue and fibrin clot to a faint pink. Harris's hematoxylin was used as a counterstain. Some of the cultures were embedded in colloidin and stained in Ziehl-Neelsen carbolfuchsin and eozin-azure after the method of Maximow. This method was afterward abandoned for paraffin sections.

Description of Living Cultures

In all, about 200 cultures were grown and observed, and about 70 were fixed and sectioned. The medium used was found suitable for the growth of guinea pig spleen.

Almost immediately after explantation cells began to migrate into the surrounding medium. These consisted mainly of leucocytes, chief among which was the polymorph. Within two hours each explant was surrounded by a dense zone of such migrated cells. The migration range was upward to 2 mm. They showed a remarkable affinity for the Johne's bacilli, and, where clumps of the organisms were within migration range of the explant, they were surrounded by dense masses of leucocytes. The cells arranged themselves about each clump in an almost solid wall, completely obscuring the clump of organisms. No similar clumping was seen in uninoculated cultures although after a greater lapse of time a few cells were seen to be adhering to each other. It was noticed that the granules in many of the leucocytes surrounding such clumps exhibited a very pronounced Brownian movement not observable in the same type of cell at some distance from the clump nor by cells in the control cultures. At the same time it was seen that many of these cells were swollen and more vesicular than others. Those in closest proximity to the organisms soon lost their ameboid movement and were apparently dead. There is little doubt but that these changes consisted of cloudy swelling and an accompanying hydropic degeneration, and the accelerated movement of the granules was due to the fact that the cyto-

plasms of the cells were of lesser density due to the imbibition of water.

This active migration of leucocytes was observable during the first 24 to 36 hours, after which these cells, although still present in the culture, degenerated rapidly, lost most of their activity, and were obscured by an active migration of macrophages from the explant. The latter arise both through the transformation of lymphocytes and from the reticular tissue. These cells at first migrated forth from the explant at a somewhat slower rate than the polymorphonuclear leucocytes. They in turn surrounded any clump of organisms within their field of migration and apparently ingested the dead leucocytes and cell debris as well as organisms which they encountered. They could be seen accumulated in large numbers around clumps of bacteria, and ofttimes it was observed in the live culture that their cytoplasms fused, forming a multinucleated or giant cell. This phenomenon of cell fusion was seen more frequently in the fluid covering the culture or in areas in which the solid fibrin clot had been digested. Ofttimes very large cells containing 30 or 40 nuclei were seen closely adhering to the coverglass in such liquefied areas. The latter type of cell was seen not only in the inoculated cultures but also in the controls.

Wherever macrophages encountered a clump of bacilli, they paused to phagocytose them, working themselves into the clump and gradually breaking it up and ingesting all of the organisms, providing the clump were not too large (Figs 11 and 16). Wherever such clumps were encountered, the macrophages seemed

to lose much of their motility, retract their many pseudopods, and become, for the time being at least, round, dark cells (Figs 6, 9, 10, and 11). Many of these clumps upward to 50 or 100 u. in diameter may be seen in a heavily inoculated culture.

At the same time the macrophages were migrating forth, fibroblasts sent out their spear-like processes into the surrounding medium. These did not migrate, nor did they become isolated from the main explant but always grew out in a dense mass and seemed to retain their connection with the main explant. No significant reaction on the part of the fibroblasts was observed in the inoculated cultures. Their growth seemed to be somewhat more profuse in the controls. However, it may be that there was a stimulation of the growth and migration of macrophages which made the fibroblastic growth seem less by comparison.

Description of Fixed Cultures

An examination of fixed, sectioned, and stained cultures discloses that the polymorphonuclear leucocyte is the first cell to meet the organisms. In cultures of a few hours growth it may be seen that leucocytes in large numbers, among which the polymorph predominates, have surrounded the clumps of organisms. It is evident that such contact soon destroys the polymorphs for it is difficult to find any around the clumps that are not already disintegrated in five hour cultures. Occasionally intact polymorphs are seen near the

periphery of such groups, and sometimes organisms are seen within their cytoplasms. It would seem that it is not necessary for the polymorph to engulf rods to destroy them. Some clumps are seen where it is observable that those rods near the periphery of the clump have lost their acid-fast staining quality while those in the center still retain it. It is believed that such digestion is the result of lytic ferments released by the disintegrating cells. However, when the polymorphonuclear leucocyte encounters isolated organisms or small clumps, the rods are ingested and may be seen in considerable numbers within an apparently intact cell. Occasionally acid-fast granules are also seen within such isolated cells.

The polymorphonuclear leucocytes in contact with organisms soon disintegrate. Those at some distance from any large clump retain life somewhat longer but soon lose their motility and with it probably their phagocytic activities. Lymphocytes in small numbers are seen surrounding clumps, and changes indicating that they become transformed into macrophages are seen. Some also are degenerated. Macrophages that are in the tissue as such before transplantation also migrate forth and exhibit an affinity for Johne's bacilli similar to the polymorph. They generally ingest bacilli wherever they are encountered.

Reticular cells are transformed into macrophages and begin to migrate forth after about 36 to 48 hours; lymphocytes which have already migrated from the explant are also transformed into macrophages. They ingest the dead leuco-

cytes and cell debris and often contain granules of pigment in their cytoplasms. They quickly ingest and replace the dead polymorphs around the clumps of bacilli (Fig. 13). Their cytoplasms often fuse with those of adjacent macrophages and form a complete encircling barrier around the clumps. On the inner surface of the sphere thus formed their cytoplasms are blended into the colony so that it is difficult to distinguish between the cellular cytoplasm and bacterial clump. These macrophages ingest bacilli with great voracity as do also scattered macrophages as they encounter isolated bacilli. The cells force their way into the clump until the entire colony is intracellular, and the cytoplasms of the cells meet and fuse in the interior of the mass, forming a typical multinucleated cell with a peripheral distribution of nuclei similar to that seen in the bovine disease.

The organisms appear both in the clumps and intracellularly as single rods or as chains up to 12 u. in length. Although an occasional acid-fast granule or chain with a beaded appearance is seen within the macrophages as within polymorphs, it is difficult to say that there is positive evidence of digestion or destruction of organisms within these cells. A comparison of cultures five days old and thirteen days old shows no further evidence of digestion in the older cultures; on the contrary, organisms in the latter show a larger number of long chains and deeper staining qualities. So it is believed that the organisms suffer little harm within the macrophages but multiply within the cell in many cases. It might be that the heavy inoculation has overcome the natural

resistance of the cells in many instances. In some of the cultures where organisms were directly adjacent to the explant, rapid multiplication took place almost immediately, and in 19 hour cultures (T70) they may be seen growing into the explant so that dense masses are seen between the splenic cells, which exhibit only a feeble reaction on the part of adjacent macrophages. However, polymorphs are gathered around the edge of the explant in this location. They have evidently been attracted from the surrounding plasma. A goodly number of lymphocytes are also seen in such locations, but they exhibit absolutely no phagocytic powers. Many of them degenerate, and others are apparently transformed into macrophages and then exhibit the same phagocytic activities as those from other sources.

At no time was a yellow pigment similar to that described by Maximow (12) as a residue of the digestion of tubercle bacilli seen within the macrophages. Perhaps if the cultures were allowed to grow for a longer period, similar digestion might take place.

As previously mentioned, giant cells are commonly seen in the inoculated cultures and under certain stated conditions in control cultures. Many of these cells in the former contain organisms, but such is not always the case. In inoculated cultures many giant cells containing upward to 18 or 20 nuclei are seen which contain no acid-fast organisms or material. Neither are they in the vicinity of a group of organisms of appreciable size.

Fibroblasts and endothelial cells grow forth from the

original explant, and the latter often form new capillary sprouts or become transformed into cells apparently identical with the fibroblasts. No significant action on their part is seen in inoculated cultures. In some cultures bacilli up to five or six in number are occasionally seen in the cytoplasm of fibroblasts, however, no particular significance is attached to this finding for it is observed in only a few cells and, therefore, is probably accidental.

There follows a description of a few typical, fixed cultures which were allowed to grow for various lengths of time.

Culture T41. Inoculated at time of transplantation. Fixed in modified Zenker's formol after five and a half hours growth. This culture shows many polymorphonuclear leucocytes that have migrated from the explanted tissue. They show marked affinity for the clumps of organisms in the surrounding medium and are collected around such clumps in large numbers wherever they are near the explant. The polymorphs surrounding the clumps are in various stages of disintegration, with fragmented and pyknotic nuclei. They form a dense encircling mass about the clumps, and an occasional cell may be seen to have penetrated to its very center. An occasional polymorph near the periphery of the encircling cells seems to be uninjured, and a few of such cells contain phagocytosed organisms and acid-fast granules. One large clump of Johne's bacilli surrounded by many polymorphs shows a distinct peripheral ring containing no observable organisms and very little acid-fast material while the center of the clump shows the usual acid-fast rods. It appears that the organisms in this peri-

pheral zone have been lyzed by the ferments liberated from the disintegrating polymorphs. Other clumps illustrate this less distinctly. The same phenomenon is not seen in clumps of organisms unsurrounded by leucocytes. The lysis is most observable in clumps near the explant, around which the most leucocytes have collected.

Lymphocytes and mononuclears have also migrated from the explant but at a somewhat slower rate than the polymorphs. They are occasionally seen among the cells collected about the groups of organisms. The lymphocytes do not contain organisms, but the mononuclears have actively phagocytosed Johne's bacilli. There are several giant cells, the nuclei of which resemble those of small lymphocytes. They contain no organisms. The original explant shows a hypertrophy of fibroblasts and an accumulation of lymphocytes at the edge. No actual growth of organisms is observed in the culture.

Culture T70. Inoculated at time of transplantation and fixed in modified Zenker's formol after 19 hours growth. This culture displays attraction of cells to the clumps of Johne's bacilli similar to that described in T41. The polymorph is still the predominating cell around such clumps, but lymphocytes and also large mononuclear cells with small, dense, eccentrically located nuclei are seen in smaller numbers. Cells of reticular origin are seen around several groups of organisms located at the very edge of the explant, from which there is an extension by growth into the explant. The Johne's bacilli of these clumps have apparently excited as yet very little reaction on the part of the cells except

slight polymorph and mononuclear attraction at the border of the colony farthest from the explant. Only rarely can organisms be seen within those polymorphs that are collected around the larger clumps of organisms. In such locations rods can be seen more frequently within the rather large, vesicular mononuclear cells. However, those isolated polymorphs that appear throughout the medium can frequently be seen to contain Johne's bacilli. Slide 13 shows many beautiful examples of phagocytosis by polymorphs, and as many as five to seven organisms are seen within one cell. Many of these cells show but little evidence of degeneration. Others, however, show a fragmentation of the nucleus into five or six distinct dark-staining chromatin granules. The organisms in those clumps at some distance from the explant, surrounded by leucocytes, mainly polymorphs, differ greatly from those in several colonies occurring at the very edge of, and extending into the explant, around which are a few macrophages and a number of fibroblasts and lymphocytes. The former clumps show only scattered acid-fast rods occurring in short lengths and beaded chains, and these mainly in the center where they are less exposed to leucocytic ferments. The rods of the latter described colonies, however, occur in dense masses near the periphery of the explant and take a deep acid-fast stain. As they extend into the explant, they become more scattered. In short, it appears that those at some distance from the explant are being rapidly lyzed by the surrounding leucocytes while those occurring near the explant find conditions favorable for growth and are multiplying unchecked by

cellular reactions. An occasional macrophage containing bacteria is seen. The maximum migration range for tissue cells in this culture is about 1 1/4 mm. An occasional mitotic figure is seen.

Cultures T73 and T76. Inoculated at time of transplantation and fixed in modified Zenker's formol after 96 hours growth. Both cultures display many scattered macrophages which are packed with acid-fast organisms occurring in chains up to 10 u. in length. Most of these macrophages are well rounded off, and the nucleus is located at one side, apparently crowded there by the large quantity of organisms within the cytoplasm. The periphery of the explant where new cells are just growing out seems to be the selected place of growth for Johne's bacilli and offers the best picture of the pathology of Johne's disease in vitro. After a close examination of the intracellular organisms, it is difficult to say that there is positive evidence of destruction of organisms within the macrophage and giant cell. On the contrary, one gets the impression that they are multiplying. Scattered polymorphonuclear leucocytes containing rods are still to be seen. Most of such cells show degenerative changes. The organisms in many of them take a very faint stain, and some acid-fast granules can be seen. Mononuclears with small, dense nuclei also contain organisms of similar appearance. There are a few mitotic figures. Phagocytosis of polymorphonuclear leucocytes by macrophages is well illustrated in slide T73-8 (Fig. 24).

Culture T56. Inoculated at time of explantation and fixed in modified Zenker's formol after 132 hours growth.

The macrophages in this culture have been very active. They have migrated widely into the medium and phagocytosed practically all of the Johne's bacilli within migration range. Many multinuclear cells containing large numbers of bacteria are seen (Fig. 15). The greatest number of multinucleated cells occur either in the liquid or in the fibrin at the periphery of an area in which the fibrin has been liquefied. Giant cells containing no organisms are also present. The scattered lymphocytes remaining in the center of the explanted tissue are practically all necrotic. This culture illustrates well the greater tendency for fusion of cells into giant cells in the liquid portions of the media. The growth of fibroblasts is very scant.

Culture T79. Inoculated at time of transplantation and fixed in modified Zenker's formol after 12 days growth (288 hours). In many places throughout the medium colonies of Johne's bacilli up to 1/2 mm. in diameter are seen. Rods, quite thick and taking a dense acid-fast stain, may be seen as chains up to about 12 u. in length. This is in marked contrast to another culture of the same age (T77), in which the original clumps that were inoculated into the culture can be seen, but only a few rods survive out of the rather large mass. At the borders of the explant several groups of macrophages and many scattered ones may be found containing rods, which likewise form chains up to 10 or 12 u. and take a dense stain. There are many mitotic figures in this culture, indicating a rapid multiplication of cells.

Discussion

The remarkable affinity of the polymorphonuclear leucocyte for Johne's bacilli in tissue cultures is to our knowledge a phenomenon previously unrecorded by any observer. Smith (14) states that polynuclear cell invasion is not observed in any stage of the disease. In work previously done at this institution (15) it was observed that polymorphs were frequently associated with macrophages containing bacilli, particularly in the glandular tubules of the intestinal mucosa, although phagocytosis of bacilli by polymorphs was never observed. Phagocytosis in tissue cultures may be explained in several ways. It must be remembered that the tissue was from the guinea pig, a resistant animal in which the disease has not been studied extensively, and, therefore, it may be argued that the same condition might not prevail in the bovine or cultures of bovine tissue. It is true that in tissue cultures the polymorphonuclear cells migrated into the plasma, surrounded the clumps, and ingested organisms some time before the macrophage arrived on the scene, but, even in those cultures where organisms were directly adjacent to the explanted tissue, the macrophages failed to react to the presence of the organisms until many hours after the polymorphonuclear cells had done so. The question may be raised as to whether or not the polymorphonuclear leucocyte is capable of destroying Johne's bacillus or whether, on the contrary, the organisms do not soon destroy the cells and are again liberated, to be engulfed by macrophages. Observation of cells con-

taining bacilli disclosed that many polymorphs, particularly those isolated ones which had encountered and phagocytosed scattered organisms, apparently did digest them in some instances at least, for ofttimes organisms were seen within these leucocytes that stained very faintly. Others showed a beaded appearance and acid-fast granules were fairly common. On the other hand, where polymorphs had surrounded larger clumps of organisms, it was difficult to find organisms within cells. This, I believe, was due to the fact that the concentration of toxins about such clumps of organisms was so great that the cell could not long resist them and soon died and disintegrated before it had an opportunity to phagocytose rods. This is substantiated by the injury and death of the cells accompanied by the hydropic changes seen in previously described living cultures. However, where leucocytes were seen gathered about clumps in large numbers, it was observed that those organisms near the periphery of the clump and nearest to the leucocytes had apparently been lyzed by the ferments liberated from the disintegrating cells. The result was a rather sharply limited peripheral zone containing no stained organisms and a central zone where organisms had not yet been destroyed but showed injury as evidenced by beaded appearance and presence of some acid-fast granules. This is well illustrated by the camera lucida drawing (Fig. 23) and less distinctly by the photomicrograph (Fig. 22).

Although care should be used in drawing parallelisms between the reaction of cells as seen in cultures of guinea pig tissue and the disease as it exists in the bovine, it

does not seem unreasonable to assume that the polymorphonuclear cells of the bovine, in the early stages of the disease at least, possess identical power to lyze the organisms. If this be the case, why then do not the polymorphs destroy the bacilli and prevent the advance of the infection?

For an answer to this question we should turn our attention to the macrophage or epithelioid cell. Maximow and others have shown that in tuberculosis these cells arose from lymphocytes and monocytes on the field of inflammation and also from the reticular cells. When they came in contact with tubercle bacilli, they immediately ingested the organisms. Diffusible toxins also affected their progenitors, causing a transformation into epithelioid cells. Practically identical conditions prevailed in tissue cultures inoculated with Johne's bacilli. However, in cultures up to two weeks old it was not possible to say that the organisms were digested within the macrophages while in Maximow's cultures, containing tubercle bacilli, digestion was well advanced in eight days. No residual yellow pigment similar to that resulting from digestion of tubercle bacilli was seen. As stated before, the organisms seemed to find the cytoplasm of the macrophages favorable to their continued growth and in some cultures apparently multiplied freely. Since, however, the disease cannot be produced in the guinea pig, it is reasonable to assume that it is the polymorphonuclear leucocyte and its ferments that play an important part in the destruction of the organism. In tissue cultures the number of such leucocytes is believed to be limited to those that are in the

tissue at the time of transplantation, and, since they are short lived, it can be seen that the supply is soon exhausted, and their effect upon the organisms is transitory.

Theobald Smith (14) in a comparison of bovine tuberculosis with paratuberculosis states that the evidence points to the absence of any diffusible toxin, and the disease progresses by contact of bacilli, which have escaped from infected cells, with fresh tissue cells. Neither cell necrosis nor fibrosis similar to that of tuberculosis is seen, and it has been suggested (17) that the resulting severe clinical disturbance is the result of interference with absorption from the intestine by the hyperplastic tissue or to causes unknown (15). Apparently there is no toxin capable of killing the macrophages produced either by the extracellular or intracellular growth of the organisms. However, in vitro it is evident that some diffusible substance capable of attracting leucocytes and of greatly damaging certain cells in the vicinity of the colonies diffused forth from the larger bunches of bacilli. It is difficult to account for the strong positive chemotaxis exerted on the polymorphs and the death of these cells in the vicinity of the colonies on any other basis. It has also been observed (14) in the bovine that, "There is an extensive phagocytosis of red cells in the spleen and a gradual conversion of the inclusions into pigment. Evidently the red cells are damaged while circulating in the capillaries of the intestinal villi." This also points to the production of a toxic substance.

Smith (14) states that in his experience all epithelioid

cells contained bacilli. Hallman and Witter (15), however, found some early cases in which, although an abundance of epithelioid and giant cells was found, the majority of them contained no acid-fast organisms. Many giant cells and, of course, macrophages were found in inoculated cultures which contained no organisms.

It was observed that giant cell formation took place in many cases where macrophages surrounded and ingested organisms as the result of the fusion of cytoplasm of adjacent macrophages (Figs 11 and 18). As previously stated this phenomenon takes place with more frequency in liquid media where the cells are deprived of any supporting framework of fibrin. This may be due to the fact that in such medium the macrophage becomes a rounded cell, retracting its many pseudopods, assuming a non-motile state, and possibly at the same time losing its undulating membrane (19), which is probably an effective obstacle to cell fusion. There may also be an accompanying change in surface tension. Some observers have advanced the theory that cell fusion is the manifestation of a mild cell injury resulting in a fusion of cytoplasm for mutual protection. Others believe that the process of giant cell formation is essentially one of incomplete division. In the cultures herein described apparently in most instances multinucleated cells were the result of cell fusion for this process could be observed in the living cultures. However, in some fixed cultures, even after as little as 24 hours growth, multinucleated cells of a peculiar type containing as many as eight nuclei were seen. In several instances a dis-

tinct link or filament connecting two nuclei was seen in these cells. This might be interpreted as nuclei in the act of amitotic division. These cells are practically identical with some observed by Maximow in the tuberculous process in vitro. In liquefied portions of cultures large nultinucleated cells with as many as 40 nuclei, containing bacilli and engulfed blood pigment, were seen closely adherent to the coverslip. Such cells are not specific, however, having been observed under similar conditions in uninoculated cultures. Lambert (18) and others observed the same type of cell in tissue cultures of spleen.

Detailed examination convinces us that multiplication of Johne's bacilli took place within the cultures. Cultures of two weeks growth show in almost every case many deep-staining rods both intra- and extracellular. Most of these occur at the border of the explanted tissue among and within the new growth of cells. The manner in which the organisms penetrated into the explant in 19 hour cultures also demonstrates growth. Dense masses of the organisms are seen between the cells, and many scattered organisms are observed around the periphery of such masses. The organisms appear as rods as short as 0.5 u. in length or as apparently unbroken chains as long as 10 to 12 u. Longer, unbroken chains are not observed. A comparison of thrifty inoculated cultures with those in which growth of the explanted tissue was scanty, because of undue exposure during manipulation or liquefaction of the plasma medium due to accumulation of metabolites, shows that the colonies of Johne's bacilli in the latter did not increase in size or in

number of bacteria. In such cultures after ten days in vitro, only a few rods retained their property of acid-fast staining out of relatively large clumps inoculated into the culture. This would indicate that growth of Johne's bacilli depends upon the active growth of cells within the culture in the immediate neighborhood of the organisms. Extracellular growth is not prominent in cultures older than eight to ten days because, when colonies of organisms occurred in locations favorable to growth, i.e., the zone of new cells around the explant, they were soon surrounded and ingested by macrophages.

Nothing was observed in any inoculated cultures to indicate that epithelioid cells and the ordinary macrophages are separate and distinct cells. They differ only in certain adaptations and a hypertrophy brought about by ingestion of organisms or a chemical stimulation due to the presence of diffusible substances in the inoculated cultures. Many macrophages containing engulfed bacilli cannot be differentiated in form or staining characteristics from like cells containing no Johne's bacilli or from those in uninoculated cultures. Those with and without bacilli apparently are stained supra vitally by neutral red with equal facility. The multinucleated cells are stained also supra vitally to about the same degree.

The suggestion that the extreme emaciation of Johne's disease is due to a simple mechanical interference with absorption has not received wide support. However, the apparent absence of positive evidence of any degenerative changes has led many to disbelieve in the formation of a toxin by the

organism. A most impressive observation is the fact that macrophages and giant cells in vitro, containing great quantities of Johne's bacilli, show absolutely no evidence of injury except possibly a temporary loss of motility. Neither is there any evidence of injury to, or digestion of the contained rods. Here, then, is what appears to be an excellent example of symbiosis, the organism living and multiplying within the cell, which seems to have adapted itself perfectly to the presence of the invader, and possibly sending forth diffusible toxins which are injurious to other cells, the most susceptible of which are certain blood leucocytes and erythrocytes. These observations in the cultures grown in vitro are in perfect harmony with the pathology as seen in the bovine disease. It is believed that polymorphonuclear leucocytes are attracted to the site of infection and are soon destroyed by the toxins coming from the intracellular organisms. They disintegrate and evidently liberate ferments highly lytic for the invading organisms. However, apparently these lytic ferments seldom, if ever, reach the organisms. Therefore, it would seem that they are protected by the cytoplasm of their host, the macrophage or giant cell. It is possible that erythrocytes circulating through the capillaries of the intestinal mucosa and mesenteric lymph glands encounter these same toxins and are gravely injured by them, so much so that their usefulness is impaired and they are consequently engulfed by the phagocytosing cells of the spleen when they reach that organ.

Since absorption in the intestinal tract is in all probability a vital process carried on by cells of the intes-

tinal epithelium, it may be that this function is likewise interfered with by the same toxin. If the above hypothesis is correct, this, together with the injury to blood leucocytes and erythrocytes, would serve to explain the extreme emaciation observed in many bovine cases. This hypothesis assumes the ability of the toxin to diffuse forth from the macrophage and the inability of the lytic ferments to penetrate into the same cell, a not unreasonable conclusion in view of the fact that uninjured cells within the body are able to protect themselves from heterolytic ferments liberated from other cells of the organism.

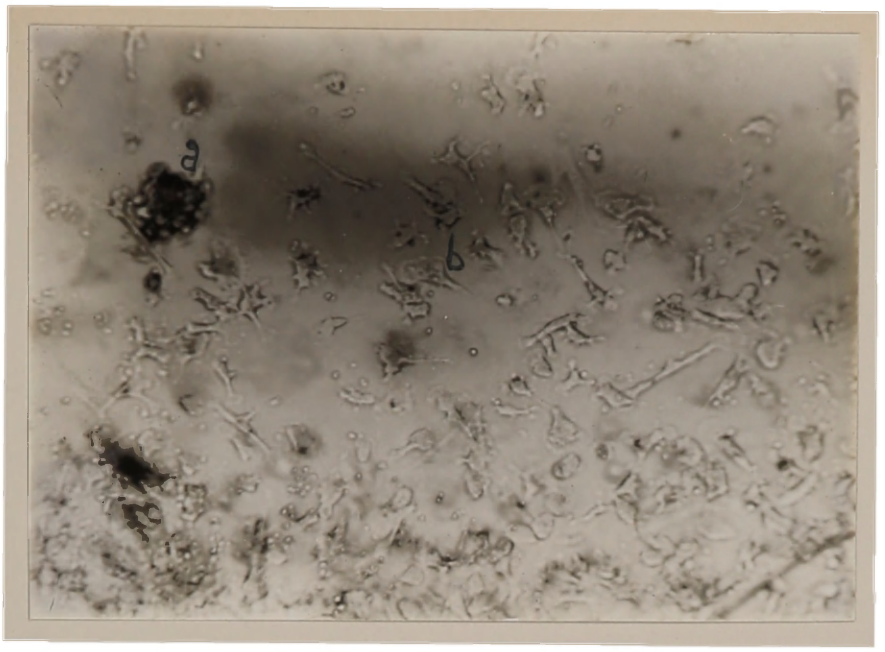
Since Johne's bacilli were apparently able to multiply within the macrophages of guinea pig tissue in vitro, we may conclude that the resistance of the guinea pig must depend upon some systemic, undetermined factor not operable in the cultures grown in vitro or, as stated before, to a more efficient phagocytosis on the part of the polymorphonuclear leucocyte.

Summary

1. Mycobacterium paratuberculosis was inoculated into cultures in vitro of guinea pig spleen. The organisms survived and apparently grew both intracellularly and extracellularly in such cultures.
2. Evidence is presented which indicates that the polymorphonuclear leucocyte is capable of phagocytosing and digesting the organism intracellularly and that ferments liberated by the disintegrating polymorphonuclear leucocytes apparently lyze nearby Johne's bacilli.
3. A diffusible substance, strongly chemotaxic for and toxic to polymorphonuclear leucocytes, was liberated by the Johne's bacillus.
4. Macrophages and giant cells engulfed the organisms wherever they encountered them but apparently had little power of lyzing the engulfed rods. On the contrary, the Johne's bacilli seemed to find the cytoplasm of such cells a favorable medium of growth.
5. In the cultures herein described giant cell formation took place by cell fusion and, in some few instances, apparently by nuclear division. Such cell fusion took place with greater frequency in liquid medium.

Fig. 1. (above) Tissue culture of 5 days growth, inoculated with Johne's bacilli at time of transplantation. (x16)

Fig. 2. (below) A 5 day uninoculated tissue culture showing profuse growth of fibroblasts. (x160)



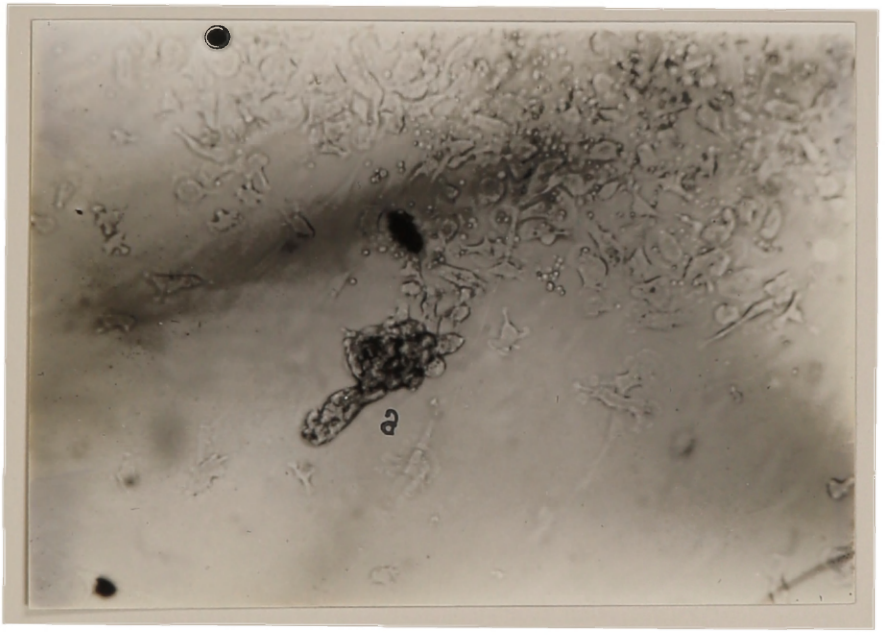
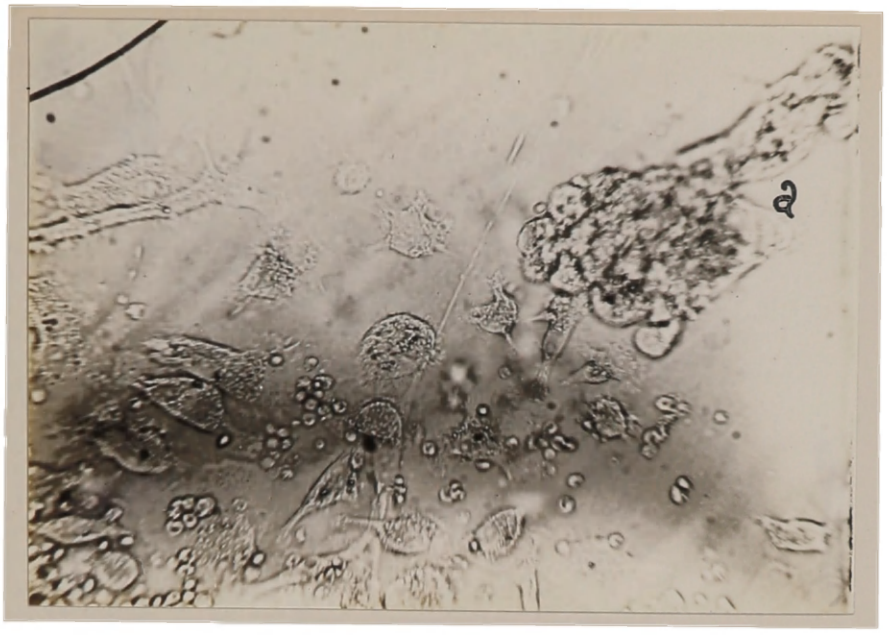


Fig. 3. (above) Illustrating a group of macrophages (a) mobilized around a clump of Johne's bacilli, also (b) scattered macrophages. (x160)

Fig. 4. (below) Giant cell formation (a) in an inoculated tissue culture. (x160)



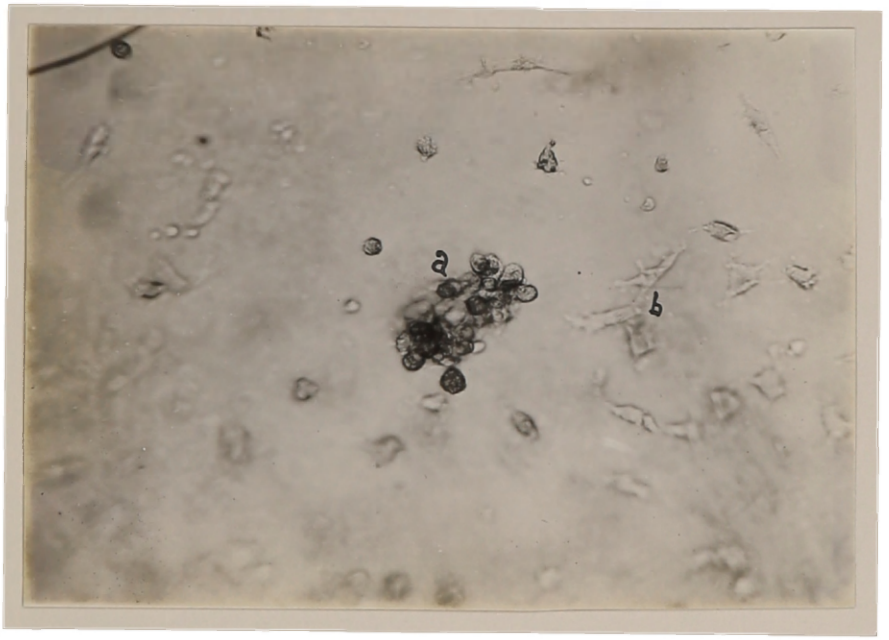
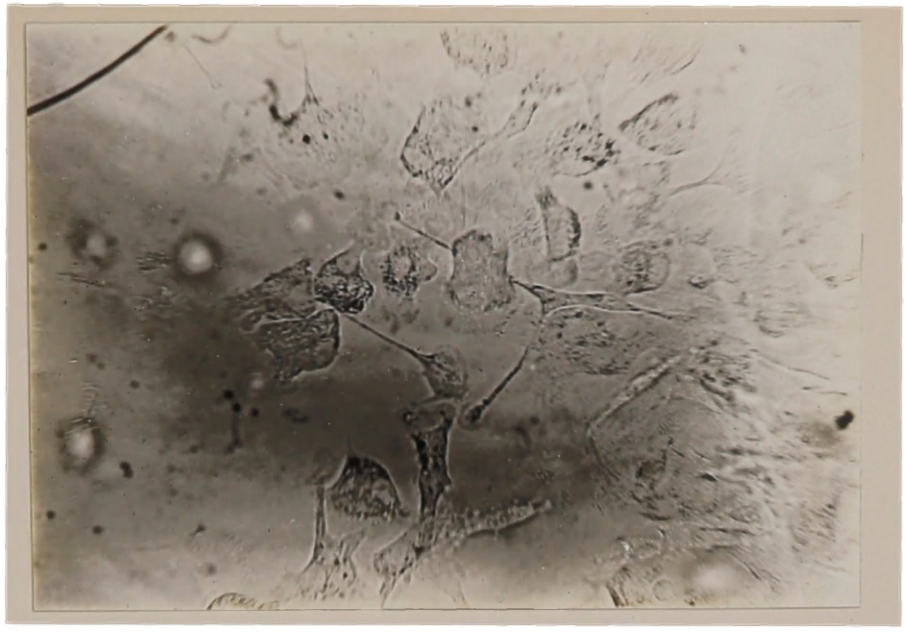


Fig. 5. (above) Giant cell formation (a) in an inoculated tissue culture. (x230)

Fig. 6. (below) Clumping of macrophages around organisms (a), the first step in the formation of giant cells. Compare these clumped cells with the scattered macrophages (b). (x160)



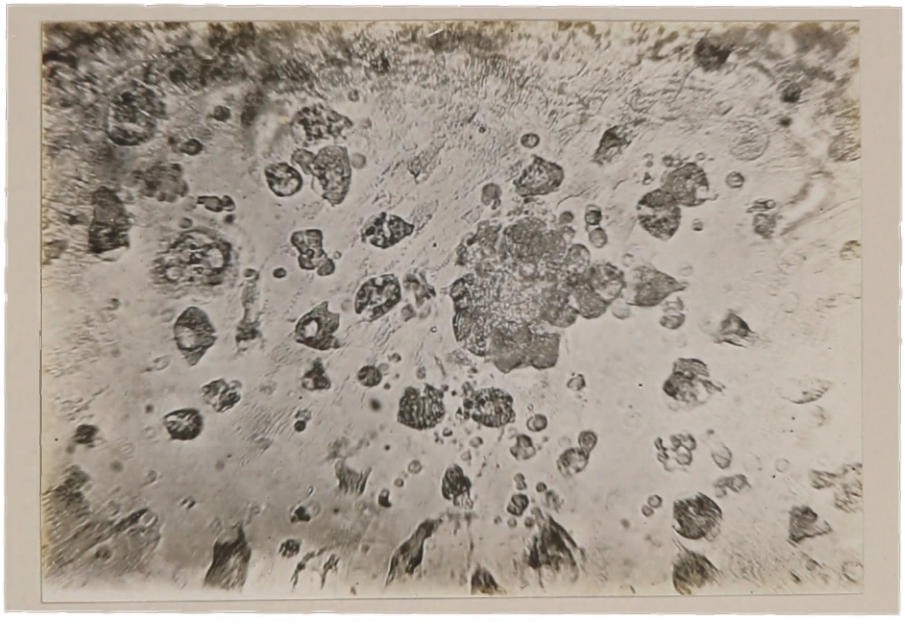


Fig. 7. (above) Macrophages (epithelioid cells) with long pseudopods in an inoculated culture. (x230)

Fig. 8. (below) Epithelioid and giant cells which have attached themselves to surface of coverslip of the culture. (x230)

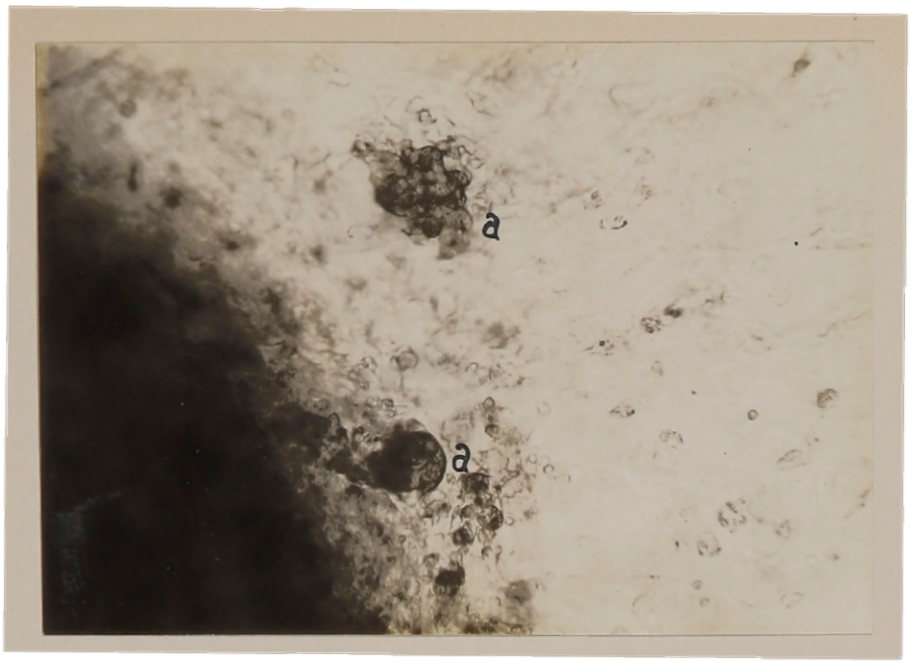


Fig. 9. (above) Mobilization of macrophages around groups of organisms at periphery of explant in inoculated culture (a). (x160)

Fig. 10. (below) At (a) macrophages are mobilizing around clumps of organisms at periphery of explant, illustrating the attraction of macrophages to the organisms. (x230)

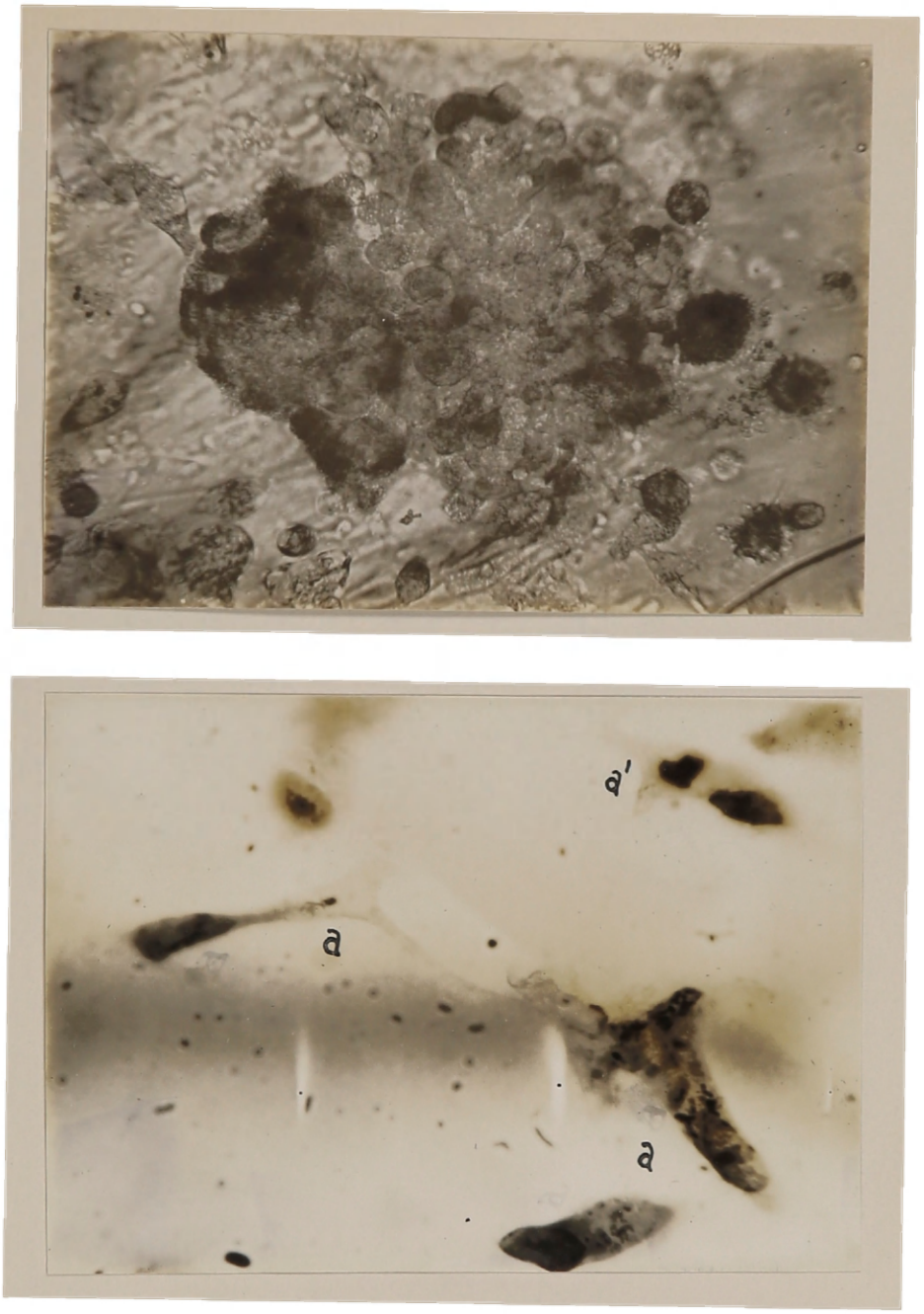
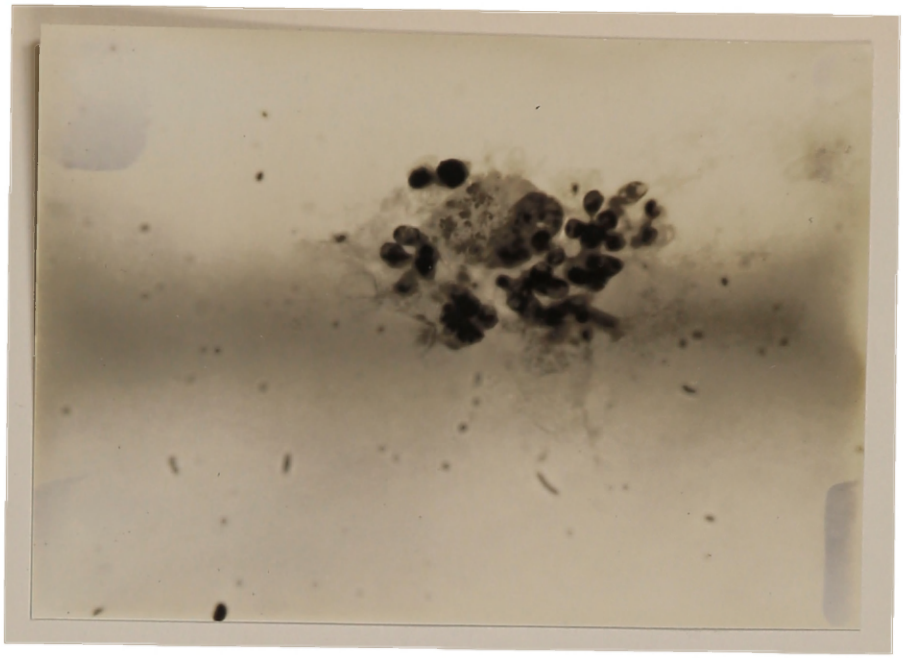


Fig. 11. (above) High power view of the mobilization of macrophages around a colony of organisms. Giant cells may result from the fusion of such mobilized cells. (x430)

Fig. 12. (below) Paraffin section of tissue culture. Macrophages (a) containing organisms. One of them (a') is in act of mitosis. Acid-fast stain, hematoxylin counterstain. (x930)



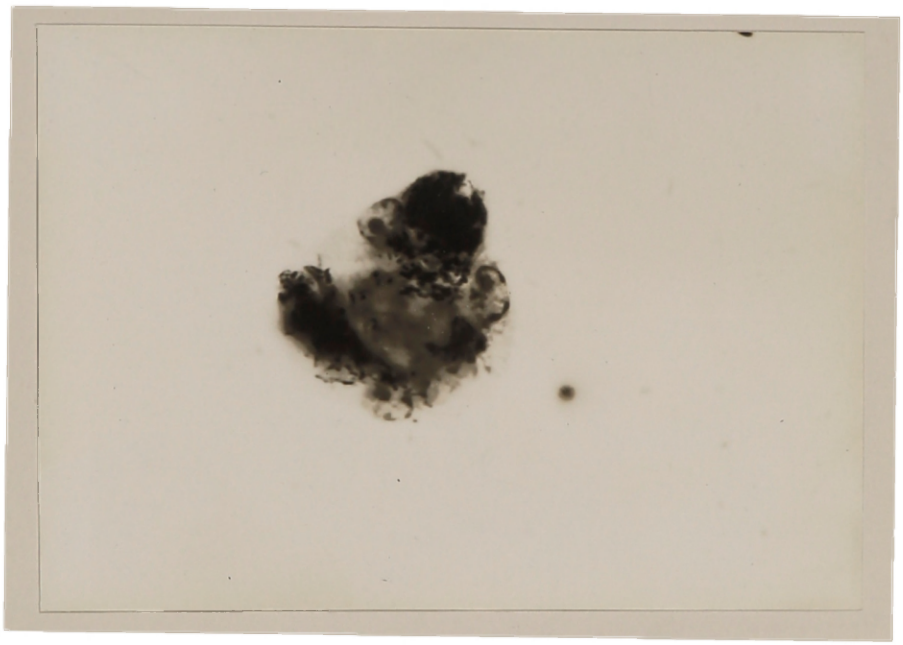
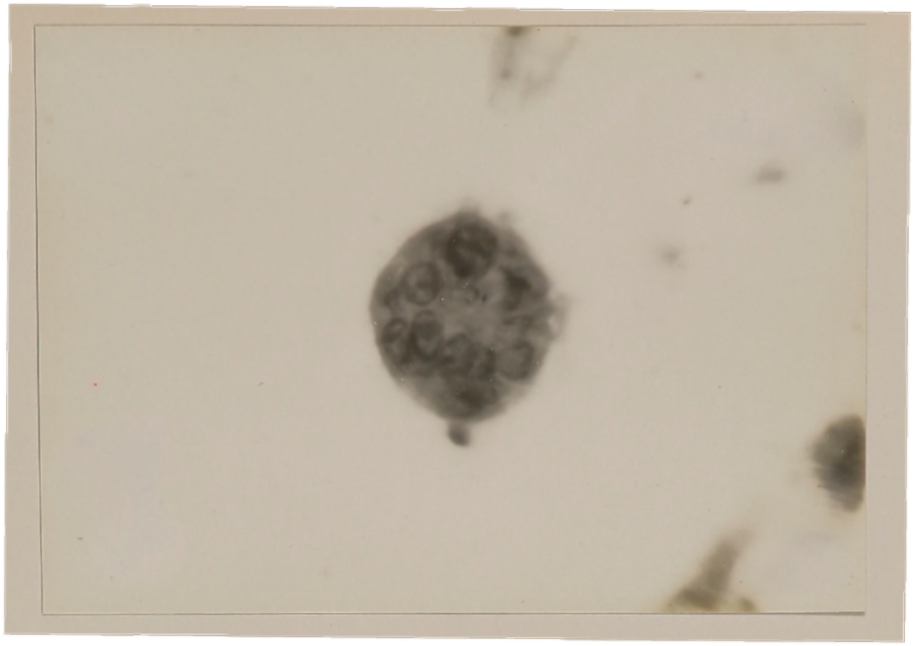


Fig. 13. (above) Paraffin section of tissue culture after 6 days growth in vitro. Phagocytosis of degenerated polymorphonuclear cells by a macrophage. Acid-fast stain, hematoxylin counterstain. (x930)

Fig. 14. (below) Paraffin section of tissue culture. A giant cell containing large masses of proliferating Johne's bacilli. Acid-fast stain, hematoxylin counterstain. (x930)



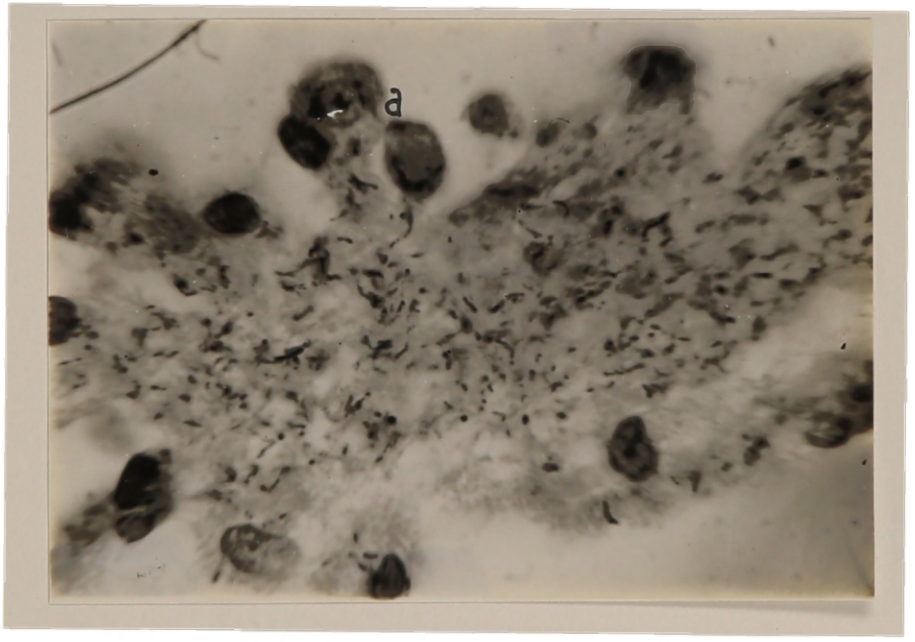
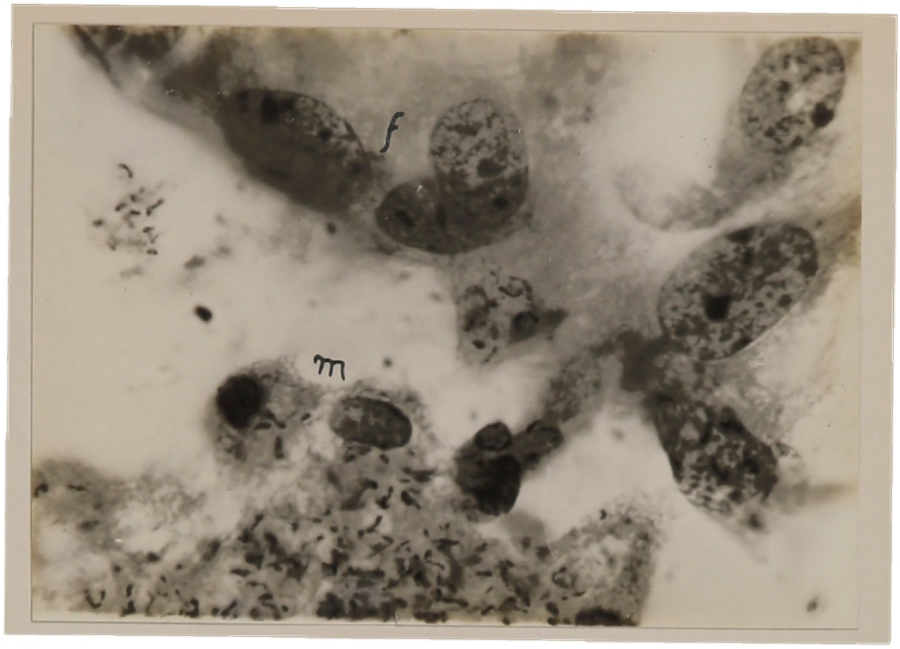


Fig. 15. (above) Paraffin section of tissue culture. A giant cell at the edge of a liquefied area of the fibrin clot. It contains a few acid-fast rods which do not show in the photomicrograph. Acid-fast stain, hematoxylin counterstain. (x930)

Fig. 16. (below) Paraffin section of tissue culture. Mobilization of macrophages (a) around a clump of bacilli at periphery of explant. The cytoplasms of the cells blend with the mass of organisms, and cell fusion often takes place to form giant cells. Acid-fast stain, hematoxylin counterstain. (x930)



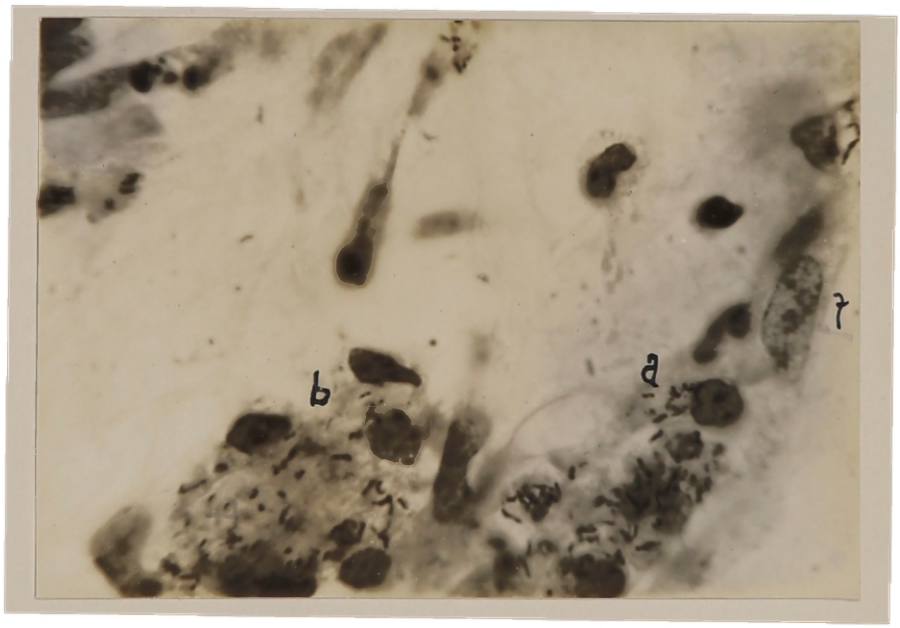


Fig. 17. (above) Paraffin section of tissue culture. Macrophages in act of phagocytosing Johne's bacilli. Note bacilli within cytoplasm of macrophages (m); fibroblasts (f). Acid-fast stain, hematoxylin counterstain. (x930)

Fig. 18. (below) Paraffin section of tissue culture. Phagocytosis of bacilli by macrophages (a). Cytoplasms of some of the macrophages have fused, forming a giant cell (b). Fibroblasts (f). Acid-fast stain, hematoxylin counterstain. (x930)

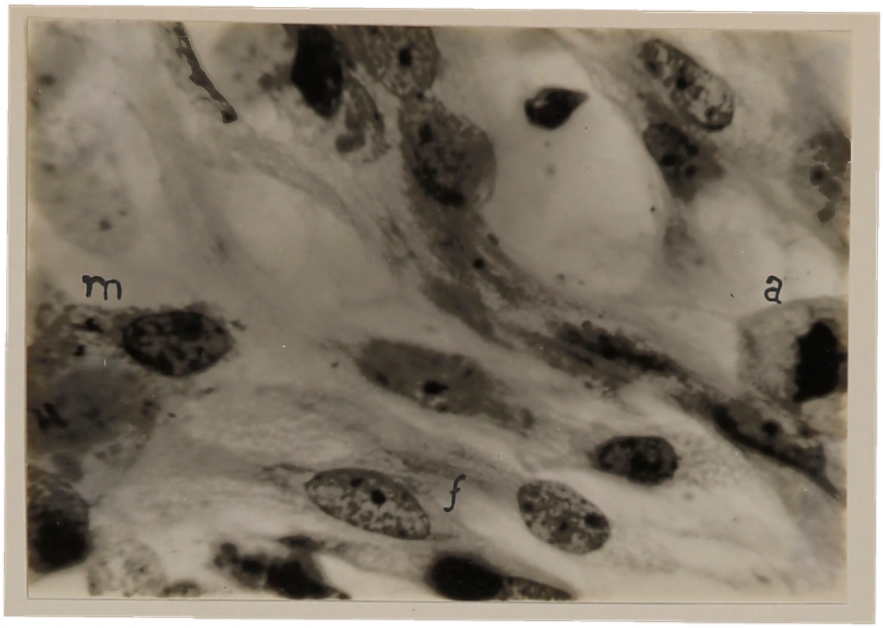
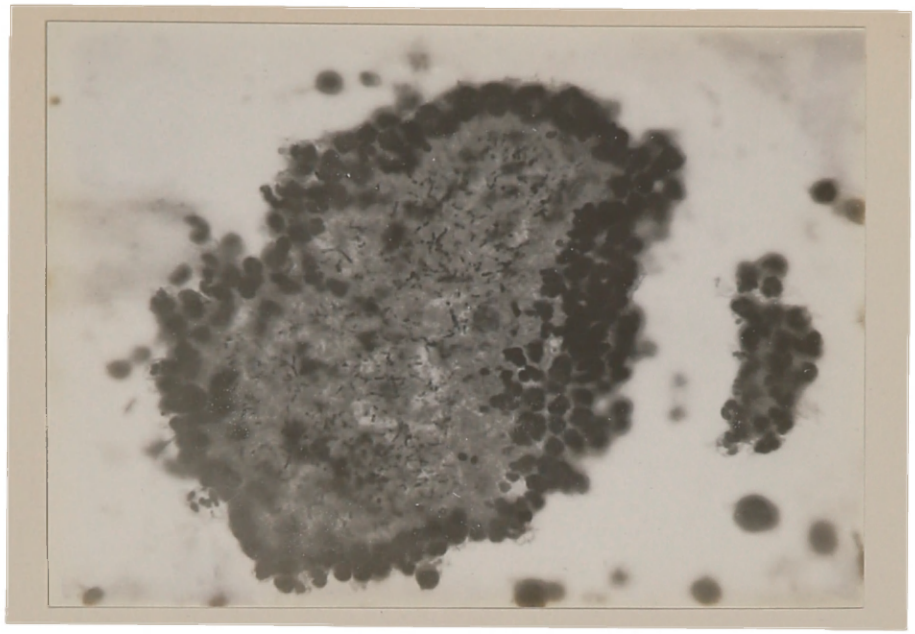


Fig. 19. (above) Paraffin section of tissue culture. Cell growth in new tissue zone around explant. Fibroblasts (f); mitotic figure (a); and macrophages (m) containing bacilli. Acid-fast stain, hematoxylin counterstain. (x930)

Fig. 20. (below) Paraffin section of tissue culture. Fibroblasts (f) in zone of new tissue at periphery of explant. Fibroblasts (f') with Johne's bacilli within cytoplasm. Hematoxylin stain, hematoxylin counterstain. (x930)



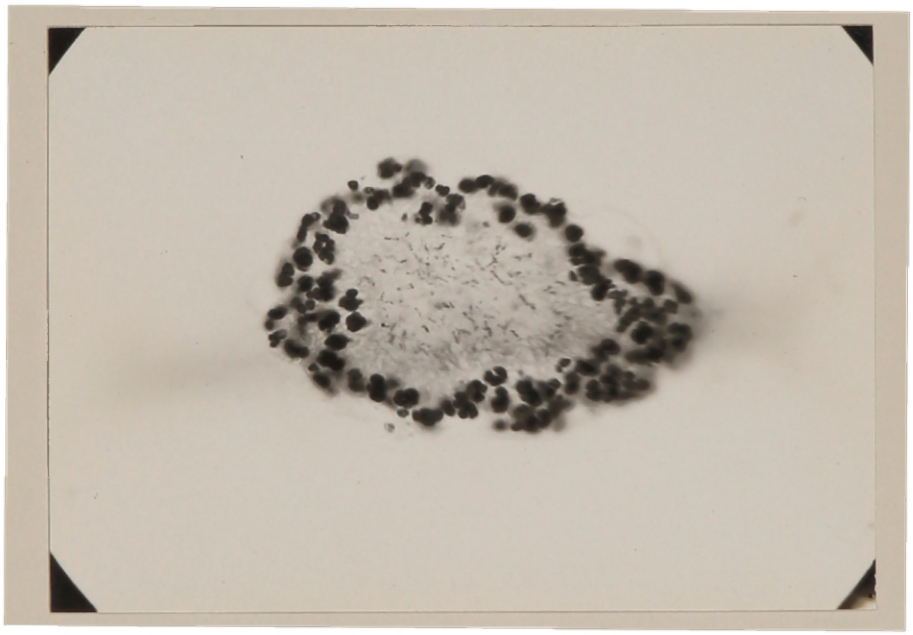


Fig. 21. (above) Paraffin section of a 5 1/2 hour tissue culture, illustrating a clump of Johne's bacilli surrounded by polymorphonuclear leucocytes. Acid-fast stain, hematoxylin counterstain. (x930)

Fig. 22. (below) Paraffin section of a 19 hour tissue culture, illustrating a clump of Johne's bacilli surrounded by polymorphonuclear leucocytes. Bacilli at edge of clump have been lyzed by ferments liberated by disintegrating polymorphs. Acid-fast stain, hematoxylin counterstain. (x620)

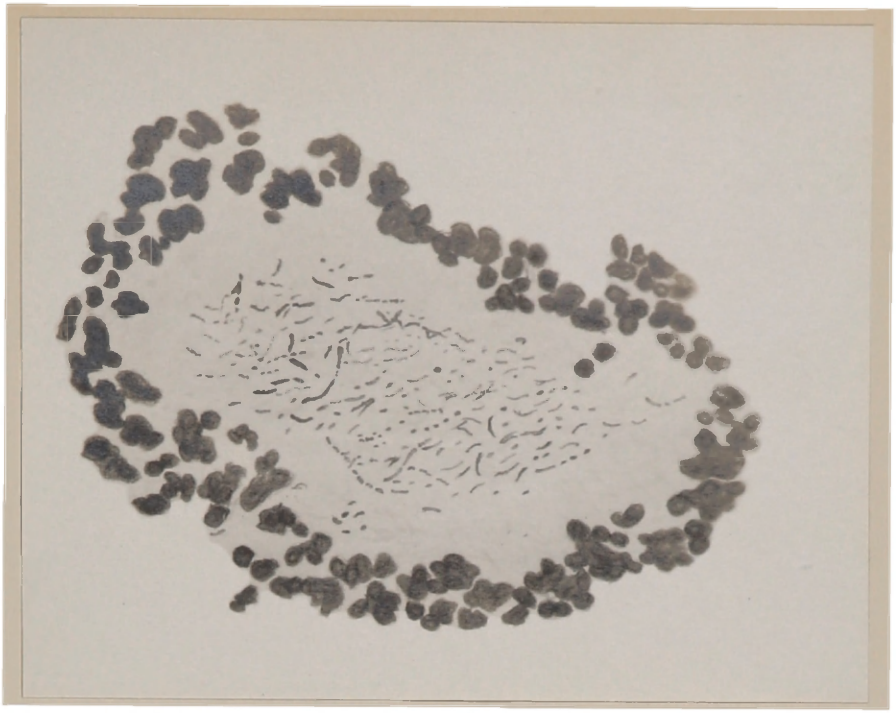


Fig. 23. (above) Camera lucida drawing of Fig. 22, showing peripheral area in which organisms have been lyzed. (app. x1000)

Fig. 24. (below) Camera lucida drawing of a paraffin section, illustrating phagocytosis of Johne's bacilli by polymorphonuclear leucocytes.
(a) Polymorphonuclear leucocyte containing Johne's bacilli.
(b) Extracellular Johne's bacilli.
(c) Intracellular organisms.
(d) Disintegrating polymorphonuclears surrounded by a few Johne's bacilli.

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