THE REACTION OF SPLENIC TISSUE IN CULTURE TO *LISTERIA MONOCYTOGENES*

By

ESTHER M. SMITH

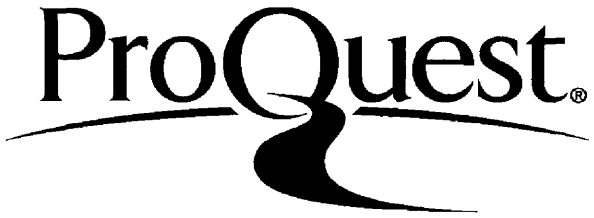
A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

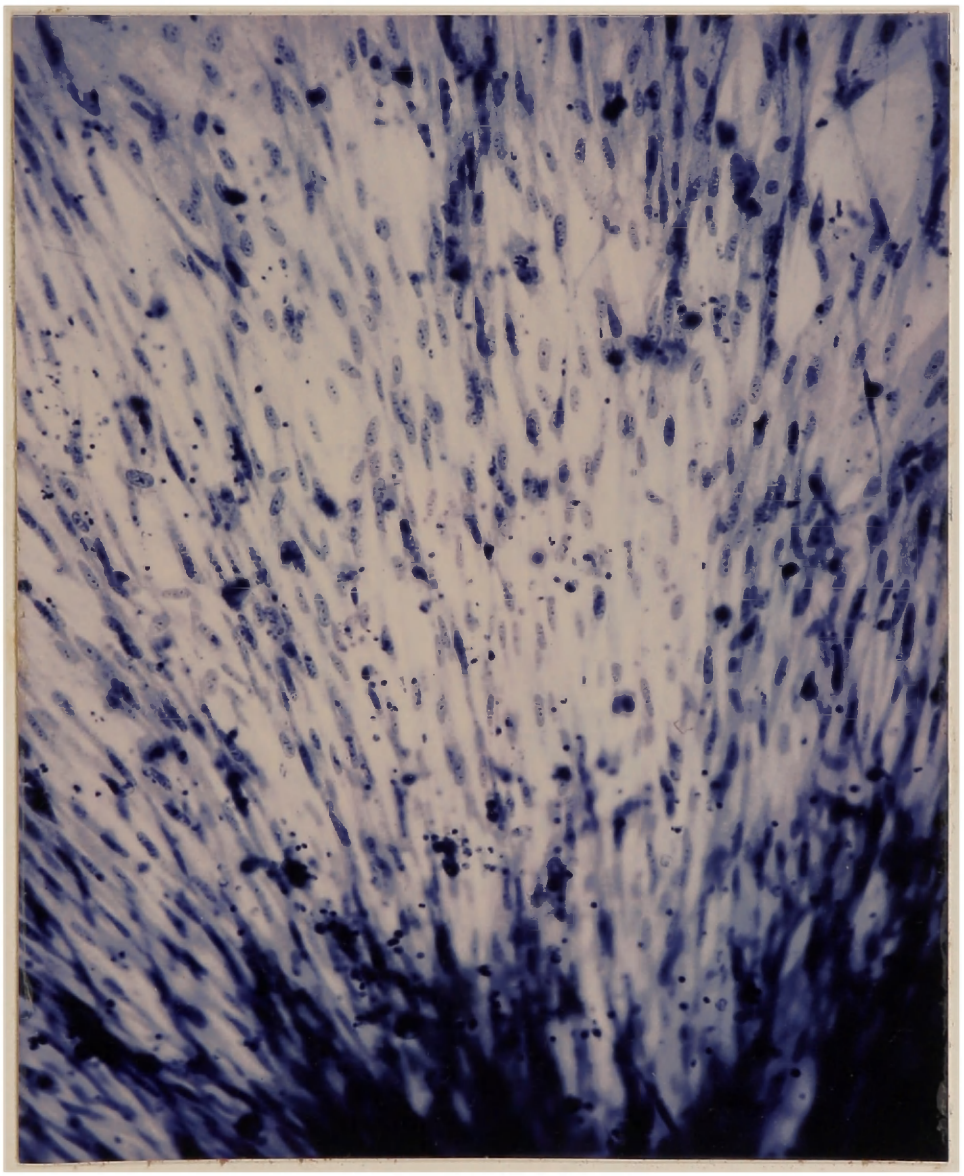
Department of Animal Pathology

1955



Frontispiece

A seven-day-old culture of spleen.
Stained with hematoxylin-eosin.



ABSTRACT

The study of tissue *in vitro* inoculated with *Listeria monocytogenes* was undertaken because of an apparent difference in tissue reaction resulting from infection with this organism. Tissue cultures provided a medium whereby the development of the lesion could be observed under controlled conditions.

Explants of spleen were placed in plasma clots, inoculated with *L. monocytogenes* and sealed in depression culture slides according to the method of Maximow. The bacterial suspension was made to match the 0.5 tube of the McFarland nepholometer when compared in a photelometer. Approximately 0.01 ml of this suspension was placed in each tissue culture. Control cultures were treated in a similar manner, omitting the bacteria. Medium 199 was the fluid used throughout this study.

Observations of living tissue cultures revealed that the growth of the tissue was not inhibited by the presence of *L. monocytogenes*. In all inoculated tissue cultures the bacteria were always motile when observed under the microscope.

When inoculated tissue cultures were placed in artificial bacteriological medium, growth of *L. monocytogenes* did not occur

unless the tissue cultures were refrigerated at 4°C for several days.

The study of stained serial sections of inoculated tissue cultures disclosed what appeared to be a proliferation of the vascular endothelium. The reaction began as early as 24 hours after inoculation and was characterized by the presence of two layers of epithelioid cells surrounding a vessel. The affected area increased in size over a six-day period. The pathological change was classified as a granulomatous inflammatory reaction. None of the uninoculated control cultures exhibited this reaction.

Further studies must be done to determine if the granulomatous reaction was specific for L. monocytogenes or if it was produced as a result of a nonspecific irritant.

ACKNOWLEDGMENTS

The author is grateful to Dr. Frank Thorp, Jr., Department of Animal Pathology, for his constant faith and understanding during the long and difficult period of study and preparation of this manuscript. He has been a very inspiring and enthusiastic teacher with whom to study and associate. The author is indebted to Dr. Mitchel L. Gray, Department of Animal Pathology, for his constant help and sympathetic attitude throughout this study. It has indeed been a pleasure to work with one who gave so freely of his time and knowledge. Many thanks to Dr. R. A. Runnells, Head, Department of Animal Pathology, for checking the microscopic sections prepared for this work and reading the manuscript.

The writer wishes to thank Dr. L. F. Wolterink of the Department of Physiology and Pharmacology for his efforts and constructive criticism of the manuscript.

It is with deep and sincere appreciation that the author extends her gratitude to Dr. M. Lois Calhoun Head of the Department of Anatomy. She has exerted a constant stimulation for creative thinking and higher learning. Without her aid, confidence, and continuous inspiration, this entire endeavor would have been impossible.

Many thanks to Dr. H. J. Stafseth and Dr. Walter Mack, Department of Microbiology, for their helpful suggestions and assistance; to Dr. Hans Ruhland, Pathologist, Michigan Department of Agriculture, for the invaluable assistance in the many German translations; to Miss Ada Beister, reference librarian, for her willingness to obtain the interlibrary loans; to Mrs. Joan VanLue; and other technical assistants, for their part in this work.

Thanks are also extended to Dr. Arnold Hooke and Charles Kupsky of Parke Davis and Co., Detroit, Michigan, for their consultation.

VITA

Esther M. Smith

candidate for the degree of

Doctor of Philosophy

Final examination: 1:00 p.m., May 13, 1955, Room 346, Giltner Hall

Dissertation: The Reaction of Splenic Tissue in Culture to Listeria Monocytogenes

Outline of Studies

Major subject: Animal Pathology

Minor subjects: Anatomy and Physiology

Biographical Items

Born: May 12, 1923, Highland Park, Michigan

Undergraduate Studies: Michigan State College, East Lansing, Michigan, 1941-1946, B.S., 1946

Graduate Studies: Michigan State College, East Lansing, Michigan, 1948-1951, M.S., 1951

Experience: Bacteriologist I, Game Division, Michigan Department of Conservation, Lansing, Michigan, 1946-1950; Bacteriologist III, Department of Bacteriology and Public Health, Michigan State College, East Lansing, Michigan, 1950-1953; on leave, 1953-1954; Graduate Assistant, Department of Animal Pathology, Michigan State College, East Lansing, Michigan, 1954-1955

Member of Society of Sigma Xi; Sigma Delta Epsilon; American Society of Medical Technologists

TABLE OF CONTENTS

LIST OF PLATES

INTRODUCTION

The behavior of living tissue cells in the presence of bacteria has always concerned the scientist. Maximow (1924) stated: "the interrelations between tissue elements and pathogenic micro-organisms constitute a problem which can be successfully attacked with the aid of tissue culture." The use of tissue culture to study the behavior of Listeria monocytogenes was undertaken in the hope of carefully controlling the reaction of the cells to this organism. The tissue culture system is free from many uncontrollable factors so often present in animal experimentation.

Since Murray, Webb, and Swann (1926) first described L. monocytogenes as the causative agent of listeriosis, the disease has been found to have world-wide distribution in domestic and wild animals. This organism is a gram-positive rod and has been confused with other microorganisms such as those of the genus Corynebacterium. It has been discarded as a contaminant, or completely overlooked in many diagnostic laboratories since many workers have reported difficulty in isolating L. monocytogenes and are quite unfamiliar with the characteristics. Gray et al. (1948) described a method whereby maceration of the tissue was followed by refrigeration and this

increased the probability of isolating the organism. This has been confirmed by many workers. Perhaps because of the confusion in identification and difficulty in isolation, the occurrence of L. monocytogenes in man has been somewhat overlooked. Very recent work in Germany indicates the possibility of a much higher incidence in man than previously reported. The microorganism has been isolated from twenty-seven species of animals and there are indications that the list of susceptible hosts will increase (Murray, 1955). The occurrence of listeric outbreaks in domestic animals may be associated with the incidence of this organism in wild animals

In addition to wide host susceptibility, L. monocytogenes manifests itself in different hosts in a variety of ways. For example, the fact that L. monocytogenes causes a severe monocytosis in rabbits but not in cattle, is in no way connected with a difference in strain. L. monocytogenes infection in goats, cattle, and sheep produces encephalitis and the organism can be isolated from the medulla oblongata. Brains of infected sheep have purulent lesions while those of cattle do not. Inoculated laboratory rabbits usually show generalized infection and exhibit focal necrosis of the liver and spleen.

L. monocytogenes often has been associated with infectious mononucleosis. The organism has been isolated from the blood of

individuals suffering from infectious mononucleosis by Nyfeldt (1929), Schmidt and Nyfeldt (1938), Pons and Julianelle (1939), and Webb (1943). However, this organism has never produced sheep erythrocyte agglutinins in the rabbit (Girard and Murray, 1951).

Abortions due to L. monocytogenes have been described in cattle, sheep, goats, swine, and rabbits (Murray, 1955). Rabbits infected during gestation always aborted after the first exposure and occasionally aborted during a succeeding gestation period as a result of this initial infection (Gray et al., 1955, 1955a). The fact that the organism apparently persisted in the reproductive tract for some time may indicate the possibility of an intracellular existence. Abortions in humans reported from the eastern sector of Germany have been attributed to L. monocytogenes and Potel (1954) described a disease of the newborn which he called granulomatosis infantiseptica. This work indicated the occurrence of a granulomatous inflammatory response.

From the foregoing discussion it is evident that L. monocytogenes and the lesion which it produces offer considerable challenge for further research. In view of the apparent differences in tissue reaction resulting from an infection with L. monocytogenes, it was suggested that tissue culture would afford a medium whereby the development of the lesion could be observed under controlled conditions.

HISTORICAL BACKGROUND

In an attempt to study cell functions and the effects of environment, Roux isolated the medullary plate of a chicken embryo in a warm saline solution as early as 1885. This was not reported until 1923 when he proved that the closure of the medullary tube was a function of its constituent cells and not of mechanical pressure from nearby structures.

In 1897, Ljunggren was able to keep transplants of human skin alive for weeks in ascitic fluid.

Loeb (1902) discovered that it was possible to obtain outgrowth of cells in plasma clots or on agar sheets placed beneath the epidermis of the ear of a rabbit. Harrison (1907) was the first to observe cell growth under the microscope. He recorded the development of nerve fibers after implanting fragments of embryonic frog neural tube in frog lymph.

Burrows (1910) observed cell mitosis in vitro and Carrel and Burrows (1910) developed the coagulated medium technique as it is known today.

It was soon realized that coagulated plasma alone did not contain all the substances necessary for continuous cell growth. Carrel

(1912) first used embryo extracts and made long-term tissue culture possible. As a result he maintained a strain of fibroblasts for over thirty years.

Lewis and Lewis (1911) pioneered the fluid medium technique by employing various combinations of salt solutions, buffer systems, and serum. Lewis (1916) used sea water as a medium for tissues in vitro.

The importance of proteins in cell nutrition was realized when Carrel (1913) discovered that extracts of tissues accelerated the growth of fibroblasts. The use of a solution containing irradiated beef plasma, Witte's peptone, hemin, cystine, insulin, thyroxine, glucose, and salts was reported in 1933 by Vogelaar and Erlichman.

Baker and Ebeling (1939) devised a fluid containing more amino acids, hormones, and nucleic acids than previous workers had reported. The nutritive and maintenance value of the medium was improved with these supplements.

The foregoing studies demonstrated the important role of proteins and protein degradation products in the nutrition of animal cells in tissue culture; and also emphasized that serum or embryo extract could not be eliminated from the medium.

Simms (1936) and Simms and Sanders (1942) reported the use of serum ultrafiltrate as a basic medium for tissue cultures. This

fluid contained a substance which stimulated the growth of dormant adult tissues in vitro.

Fischer (1946) summarized the work on tissue culture for the previous twenty years. It was apparent from this review that during this period considerable attention had been given to cellular nutrition with special reference to growth-promoting substances and the nitrogen metabolism of tissue cultures.

As early as 1926, Baker and Carrel studied the protein fraction of embryonic tissue extract and found that fibroblasts grew faster in this fraction than in others. This portion consisted of a mixture of nucleoprotein and glycoprotein. Fischer (1941) fractionated embryo tissue juice and reported that the growth-promoting factor was associated with the nucleoprotein portions and termed it embryonin.

Fischer et al. (1948), employing a mixture of biologically important substances, devised a medium known as V-605. One of the most important amino acids in V-605 was found to be cystine, since its absence prevented growth. Arginine, tryptophane, glutamine, and lysine were found to be necessary in this order of decreasing importance.

Morgan, Morton, and Parker (1950), using the building-up method, developed a very complicated medium known as 199. This

is characterized by a complete supplement of amino acids and vitamins in addition to nucleic acid constituents, purines, pyramidines, and pentoses. Tween 80 was used in this mixture to supply a source of fatty acid and aided in dissolving fat-soluble vitamins and cholesterol.

An adequate medium, completely synthetic, has not yet been devised. The elaboration of such a medium is the goal of the tissue culture worker but the attainment of this goal does not seem close at hand.

Plasma clot cultures grown on coverslips as used by Carrel (1912) provided a satisfactory method for cultivation. Sometime later, Carrel (1913a) cultivated tissues in tubes which were lined with a thin coagulum of plasma and embryo extract. This early work was the basis for the present-day roller-tube technique. However, Gey (1933) made the roller-tube a practical system of cultivation. Porter et al. (1945) devised a flask with a long straight neck to fit a roller-tube rack. Today this flask bears the name of the senior author.

Until recently the plasma clot culture was the most satisfactory method available, but there are many limitations. The clot is difficult to handle because the operator must be able to control the

clotting mechanism. The clot interferes with microscopy because of its thickness, and liquefaction occurs during cellular growth and then the clot must be repaired. Evans and Earle (1947) replaced the plasma clot with perforated cellophane. This new substrate makes possible the uniform dispersion of cells over the surface of the culture flask so that the cells can be scraped off the glass-cellophane interface and transferred as a suspension. With the cells outside the confinement of the clot, single cell cultivation was possible (Sanford et al., 1948).

The use of cells grown on glass or cellophane provides a means for accurate, quantitative work with replicate cultures (Evans et al., 1951). Nuclear counts can be made in a hemocytometer. It is then possible to compare the number of cells originally planted with counts made after incubation or experimental manipulation.

The need for suspension-cell cultures for virus propagation stimulated Simms and Stillman (1937, 1937a) to devise a method for the treatment of kidney tissue with trypsin. These authors reported that trypsin stimulated initial growth due to the removal of an inhibitory material from tissues as a result of a proteolytic action. Robbins, Weller, and Enders (1952) made a similar report.

Dulbecco (1952), and Dulbecco and Vogt (1954) described a method for trypsinizing tissue to obtain a monolayer culture to study

animal viruses. Youngner (1954) adapted Dulbecco's method to prepare trypsin-dispersed monkey kidney cell suspensions which were standardized and used for the preparation of large numbers of replicate cultures. Youngner (1954a) utilized the trypsin-dispersed monkey kidney cells for the titration of poliomyelitis virus in roller-tube cultures. This is the basic method used today for the production of the poliomyelitis virus vaccine.

REVUE OF LITERATURE

Although the behavior of tissue cells *in vitro* was studied by Carrel and others in the early part of the twentieth century, pathologists and bacteriologists made little use of the opportunity to study the reaction of tissues to bacteria in this manner.

Smyth (1915) studied the effect of *B. diphtheriticum*, *B. typhosus*, *B. coli verus*, *B. prodigiosus*, and *Micrococcus aureus* on tissue cultures of heart and spleen from chickens. The strain of *B. typhosus* was not pathogenic for chickens and it was found that the organism did not live in fresh chicken plasma. Smyth reasoned that since the organism did not remain alive, the plasma contained bactericidal properties; however, after several hours of incubation with tissue explants the organism grew well and had a special affinity for the new cells. *B. coli verus* and *B. prodigiosus*, which were nonpathogenic to the chicken, colonized and the presence of the tissue fragment did not seem to have any influence on the growth of the bacilli. In tissue cultures infected with *Micrococcus aureus*, the bacillus grew freely in the presence of the tissue, and somewhat dispersed tissue growth. Smyth (1916) elaborated on some of his earlier

experiments and described the effect various tissues exerted upon the growth of bacteria.

Lewis (1920) introduced B. typhosus into tissue cultures of chicken embryo intestine. He was unable to keep tissue cells alive much beyond 24 hours. Soon after introducing the bacteria into the cultures, large vacuoles appeared in the cells, together with an increase in their size and number. Some of the vacuoles contained bacteria which remained alive and motile within the vacuole.

Smith et al. (1922) studied the behavior of chicken embryo tissue containing avian tubercle bacilli and concluded that the cells were not attracted by the bacilli and that the presence of the organism did not induce any change in the cultures.

An outstanding piece of work was reported by Maximow (1924) on the reaction of mammalian tissues in vitro to Mycobacterium tuberculosis. Lymphoid tissue, mesenteric lymph nodes, and omentum were grown in plasma clot cultures and inoculated with one of two different strains of human tubercle bacilli. One strain was highly virulent and the other was avirulent. After the tissues had been in culture for three to five days the bacilli were added. Maximow found that the best picture of the relationship between cells and the bacteria were seen at the periphery of the explant in the

zone of newly formed tissue. Cultures which were infected with the virulent strain were able to overcome the infection and after seven to eight days only a few bacilli could be seen in the cultures. However, the avirulent strain completely overgrew the tissue cultures after three weeks in vitro. He reported that the tissue cells did not perish in any of the cultures inoculated with the virulent strain but rather displayed a series of reactive changes which duplicated the specific tuberculous inflammatory process. It was pointed out that this was in marked contrast to the behavior of cells which became accidently contaminated with saprophytic organisms, since in this case the cells were unable to resist the influence of these intruders and were soon killed.

As a result of these experiments, Maximow (1924) concluded that the reticular cells of the lymphoid tissue displayed the most significant reaction. They mobilized and formed wandering cells and macrophages which were named polyblasts. The cells migrated into the medium and phagocytized the tubercle bacilli. Epithelioid and giant cells developed and some of these cells contained a golden yellow pigment which was believed to be a residue from intracellular digestion of tubercle bacilli.

Lang (1925) inoculated lung tissue in vitro with M. tuberculosis. Lung tissue was used because it provided a tissue which was capable

of producing an exudative reaction, and contained capillary endothelium and septal cells. He claimed that the endothelium of lung tissue did not play a role but that the septal cells were the principal active elements. The latter corresponded to the mobilized histiocytes and were the phagocytic ameboid cells which engulfed the bacilli and were then transformed into epithelioid cells. The epithelioid cells were arranged in tubercle-like clusters. The tubercle bacilli in the tissue cultures influenced the elements of the explanted tissue by means of the soluble products of metabolism. In cultures of lymphoid tissues the endothelium of blood vessels assumed an embryonic form. Lang did not make any attempt to explain this last finding, but it is mentioned here because it seemed pertinent in view of the results of this work.

To study the response of leucocytes in tissue culture to a Streptococcus, King et al. (1928) used cultures of the buffy coat from blood. The organisms were grown 18 to 24 hours in broth and diluted in Tyrode's. These workers observed that if 150,000 bacteria per milliliter were present the cells did not grow beyond 12 hours. However, if only five bacteria per milliliter were added, cellular growth continued for five days. It was also reported that the periphery of the cultures infected with the bacteria was coarse and granular in

contrast to that of uninoculated cultures which had a smooth and uniform cell distribution.

Utilizing pure cultures of fibroblasts and a gram-positive saprophyte, Tannenberg (1929) reported that individual bacterial colonies grew in the center of a fibroblastic "ring" which was not sharply defined, but had finger-like appendages. Stained preparations revealed these fibroblastic cells to be filled with bacteria and he therefore assumed that the fibroblasts phagocytized the organisms. The fibroblasts containing bacteria did not take the nuclear stain, indicating the possibility of death. Cultures inoculated with bacteria did not grow well and evidence of fatty degernation was reported. As a result of these studies, it was concluded that macrophage-like cells were really degenerated forms of fibroblasts.

Aronson (1931) studied the specific cytotoxic actions of tuberculin in tissue culture. Using tuberculous guinea pigs, explants of bone marrow, spleen, and testes were cultured in the presence of tuberculin from the human and bovine type. It was found that the tuberculin was toxic to the cells, but when explants of the same tissues taken from nontuberculous animals were cultured in the presence of the same tuberculin, there was no toxic effect.

In order to study the behavior of Treponema pallidum in vitro, Földvari (1932) and Kast and Kolmer (1933) attempted to grow this

organism in the presence of living tissue. These investigators were unsuccessful in this endeavor and were unable to come to any definite conclusion as to the relationship of this organism to the host cell.

In an effort to observe the chemotropism of polymorphonuclear cells, McCutcheon and Dixon (1936) reported that these cells exhibited a positive chemotropism to Staph. albus, Str. hemolyticus, M. tuberculosis, E. typhosus, and T. histolytica.

It is well known that polymorphonuclear cells are the first to appear in the area of inflammation but monocytes also play a vital role in combating infections and removing debris. Herzog (1938) used tissue cultures of human leucocytes to study the phagocytic activity toward various agents. Coman (1940) used cultures of monocytes to compare the chemotaxis of monocytes with that of polymorphonuclear leucocytes and lymphocytes. He obtained monocytes by explanting small pieces of rat omentum. After two days of incubation, the migrating cells were mononuclear phagocytes. The polymorphonuclear cells were grown from the buffy coat of blood and lymphocytes from the rat lymph nodes. The substances which were tested for the chemotaxic effect were placed 0.5 mm from the explant and various migrations were recorded. It was concluded

that polymorphonuclear cells exhibited a positive chemotaxis to S. aureus and M. tuberculosis but were repelled by alumina silica. Monocytes and lymphocytes did not show a positive chemotaxis to either organism and monocytes showed a very slight negative chemotaxis to alumina silica.

Later investigations of T. pallidum in tissue cultures by Perry (1948) revealed that the organism survived for ten days and that giant cells were produced. This giant cell production suggested the possibility that they were associated with a granulomatous inflammatory reaction. It was also reported that T. pallidum did not grow if the tissue died.

Allgöwer and Block (1949) studied the effect of tubercle bacilli on the migration of phagocytes in vitro. These workers found that leucocytic migration was inhibited if the cultures were infected with virulent bacilli but if white cells were inoculated with avirulent bacilli this migration was not inhibited. This somewhat corroborates the work of Aronson (1931).

It was not the intention of the writer to prepare a literature review on L. monocytogenes per se; that has been done by Seeliger and Linzenmeier (1953) and Gray (1954) and any repetition here would be superfluous. Only those papers which were concerned with the reaction of the tissue to this bacterium will be reviewed.

After Murray, Webb, and Swann (1926) described the severe monocytosis produced in rabbits by L. monocytosis, many investigators began to use this organism to study the controversial question of the origin of the monocyte.

Bloom (1928) concluded that the monocyte developed by individual transformation of the lymphocytes in the blood; especially in the sinuses of the spleen and liver. Splenectomized rabbits inoculated with L. monocytogenes developed monocytes from the bone marrow. He stated that monoblasts did not exist and differentiated monocytes from free histiocytes.

Lang (1928) reviewed the literature on the monocyte question, but did not come to any definite conclusion.

Nyfeldt (1932) injected rabbits with L. monocytogenes and found no cytogenic centers for monocytes. He stated that there was no transitional process from myeloblast to monocyte but that monocytes came from the stimulation of lymphatic tissue.

Rezzesi (1933) and Wallbach (1934), studying rabbits infected with L. monocytogenes, concluded that the most important cell in this disease was the monocyte.

Conway (1938, 1939) investigated the reaction of rabbit lymphatic tissue to injections of L. monocytogenes. Microscopically the

splenic response was characterized by the movement of lymphocytes from lymphatic tissue of the periarterial sheaths into the red pulp. The rate of formation of new lymphocytes in the sheaths initially was greater than the rate of destruction. It was concluded that the lymphocytes were associated with the transitional form of monocytoid lymphocytes. The monocytes which were seen in the red pulp, developed by individual hypertrophy and transformation of the lymphocytes.

It is known that the identification and isolation of L. monocytogenes has been overlooked on many occasions. Burn (1934, 1935, 1936) reported the isolation of an unidentified gram-positive bacillus associated with meningoencephalitis. The organism was found in the blood of three newborn infants who had died of meningoencephalitis. In two of the infants the bacteria were spread throughout all organs. After much study and research, it was concluded that this organism was of the genus Listeria.

Potel (1952) in the eastern sector of Germany reported a disease of the newborn which he called granulomatosis infantiseptica and first named the causative organism as Corynebacterium infantiseptica. However, in a discussion following the presentation of this paper, Seeliger stated that he was of the opinion that the etiologic

agent of this disease was L. monocytogenes. Since that time many reports concerning the etiology and epidemiology of granulomatosis infantisepticum have been made by Potel (1953, 1954), Riess (1953), Erdmann and Potel (1953), Hahnefeld and Nisolk (1954), and Hahnefeld (1954).

The histopathology of listeriosis has not been well defined. The most commonly described lesion is necrosis involving mostly the spleen, liver, and brain. Bloom (1928a), in describing the formation of the abscess associated with L. monocytogenes infection, stated that the transformation of lymphocytes to monocytes could be seen at the periphery of the splenic abscess. He did not believe that the vascular endothelium gave rise to the exudative cells seen in the abscesses.

Jensen and Mackey (1949), studying listeriosis in cattle and sheep, reported that the changes consisted of foci located in nerve tissue proper. These foci contained plasma cells, macrophages, and neutrophils. They observed phagocytosis of the organism by the mononuclear macrophages.

Julianelle and Moore (1942) described the pathological changes in the eye as consisting of areas of focal necrosis. In every abscess which they studied there were many mononuclear cells at the

periphery and they thought this to be a strange finding since the necrosis was caused by a pyogenic organism.

Hagemann et al. (1953) described the histopathology caused by L. monocytogenes infection in the newborn. Liver lesions were characterized by large, rounded areas containing a dense capillary network and a few atrophic parenchymatous cells. The capillary endothelium proliferated and some of the cells contained fat-like droplets and desquamated nuclear fragments. These workers classified the lesion produced by L. monocytogenes according to two possible reactions and fates. One lesion was characterized by an increase in mesenchymal cells resulting in an increase in reticular cells, histiocytes, monocytes, and epithelioid cells. This was called a "granuloma" and was not seen in all cases of infection. Bacteria were seldom seen or isolated from these lesions. The second and most frequently observed lesion was characterized by necrosis. Hagemann et al. believed the necrosis to be due to the action of a toxin or to the organism itself rather than to the obliteration of the capillaries. With the beginning of the necrosis there was a centripetal infiltration of the capillary cells. These endothelial cells began to engulf the whole area. At this time the nuclei became elongated and took the form of rods, dumbbells, hooks, clubs, and

other bizarre shapes. These proliferating capillary cells which formed a proliferating "granuloma" were the same as those described in the previous type of lesion.

Simon (1953), in reporting on listeria encephalitis, described the presence of raised areas on the meninges called "disc-like" infiltrations of a granuloma character with packed histiocytes and an increase of L. monocytogenes organisms. In the area of the sulci there was a low-grade vascular proliferation. He reported the presence of "granulomas" in the outer and medial meningeal layers. Simon stated that in the periventricular area there was fluid which contained many Listeriae, and that this produced a mechanical toxic process which caused some histolysis followed by mesenchymal cell proliferation. This new cell formation produced the granuloma or "listeroma" and these formations were connected with the capillary tissue.

Hagemann et al. (1953) reported that in the stillborn, the primary proliferation was the result of increased mesenchymal reaction in which the entire connective tissue took part. L. monocytogenes was not often observed in the granulomas.

The general opinion of these German workers was that the capillary endothelium often played a vital role in the formation of

the lesion produced by L. monocytogenes and it was upon this basis that they call the disease granulomatosis infantiseptica.

MATERIALS AND METHODS

Glassware and Instruments

Most glassware and instruments were washed in Hemo-sol,* rinsed once in tap water followed by three distilled-water rinses, and sterilized in a hot-air oven at a temperature not in excess of 140° C. for one hour. Capillary pipettes with rubber bulbs were autoclaved.

Physiological Salt Solutions

Phosphate buffered saline (PBS). Buffered saline was used for preliminary washes of tissues. The water which was used as a diluent was triple distilled in pyrex and autoclaved immediately before using. The composition was as follows:

NaCl	8.0 g.
KCl	0.2 g.
Na_2HPO_4	1.15 g.
KH_2PO_4	0.2 g.
$CaCl_2$	0.1 g.
$MgCl_2 \cdot 6HOH$	0.1 g.
HOH	1000.0 ml

* Meinecke & Co., Inc., New York, N. Y.

Medium 199. The synthetic medium of Morgan et al. (1950) was used as the basic constituent of the nutrient fluid. This medium was obtained from Parke Davis and Co., Detroit, Michigan. The pH of this solution was adjusted to 8.0 with sterile $NaHCO_3$.

Plasma

Homologous rabbit plasma was used throughout this study. A normal rabbit was bled by cardiac puncture. Approximately 25 ml of blood was drawn into a 30-ml syringe containing 5 ml of a 1:500 isotonic heparin solution. This amount of heparin delayed clotting for several hours. The blood was centrifuged at a moderate speed for ten minutes. The plasma was immediately removed and stored in an ice bath.

Embryonic Extract

Embryonic extract was prepared from nine-day-old chicken embryos. The eggs were opened and the embryos aseptically removed. After first removing the eyes, to eliminate the pigment which often makes a cloudy extract, the embryos were ground in a tissue grinder and approximately 2 ml of medium 199 was added for each crushed embryo. The mixture was placed in an incubator

at 37°C for one hour, centrifuged, and the supernatant fluid removed with a pipette. The extract was diluted with an equal amount of medium 199, and the mixture was stored in a deep freeze at -27°C.

Bacterial Cultures

The culture of Listeria monocytogenes used in this study was isolated in 1951 from the brain of a calf. The bacterial culture was prepared for use in tissue culture in the following manner. The growth on a 24-hour tryptose agar slant was washed off with sterile saline. This suspension was diluted with saline and the density read in a Cenco Sheard Sanford photelometer using the 0.5 tube of a McFarland nepholometer as standard. One drop of this bacterial suspension was added to the tissue cultures from the tip of a 27 gauge needle attached to a 5-ml syringe.

Tissue Cultures

All tissue culture work was carried on in an asbestos-lined hood, located in the culture room. The hood was thoroughly washed and the area subjected to ultraviolet rays for several hours prior to the beginning of each experiment.

Procurement of tissues. A small autopsy room, separate from the culture room, was used for obtaining the animal tissues. The stainless steel bench was washed down prior to the beginning of the operation. A normal rabbit was placed ventral side uppermost on a metal tray. The hair was clipped as short as possible, the entire surface sponged several times with 70 percent alcohol, a cardiac puncture made, and the animal exsanguinated. Blood was collected aseptically to provide plasma for preparing clots. After bleeding, the abdominal surface was sponged with alcohol, the skin incised and reflected, being careful not to incise the abdominal muscles. The muscle surface was wiped with alcohol to remove any hairs which might contaminate the area. The muscles and peritonium were incised with a sterile knife, the spleen removed and placed immediately in sterile Petri dishes.

Method of culture. The spleen was washed twice with PBS to remove any surface blood, and was minced into pieces approximately 1 mm square with sharp scissors. The minced tissues were washed three times with PBS followed by two washes with medium 199. Tissues were allowed to remain in medium 199 until explanted.

The double coverslip method of Maximow (1925) was used in this study. Large cover glasses, 40 x 50 mm, were placed on sterile

black porcelain tiles and covered with the bottom or top of a sterile Petri dish. One drop of sterile water was placed on the center of the cover glass and a sterile, round, 22-mm coverslip was placed on the drop of fluid and adhered tightly to the large cover glass. The explant was placed on the round coverslip and one drop of the nutrient medium was added with a pipette. The nutrient medium consisted of one part of medium 199, one part of plasma, and one part of embryo extract. Some of the tissue cultures were inoculated with one drop of the standardized bacterial suspension as described previously. A drop of sterile, melted petrolatum was placed at each corner of the large cover glass, a large depression slide was placed over the culture and after clotting occurred, the entire preparation was inverted. The culture was sealed with melted paraffin to prevent evaporation of the fluids. After the paraffin was set, the slide was placed in a rack in an inverted position in order to provide a lying drop instead of a hanging drop. The cultures were incubated at 37°C (Plate I).

When the clot liquefied, usually in four to five days, the preparation was opened and the round coverslip was removed. The coverslip with the adhering culture was washed in fresh medium 199 for 10 minutes and placed on a sterile cover glass. Fresh plasma was

Plate I. Double coverslip culture. Mag. 2x

The explant (A) is on the round coverslip (B) which is attached to the large coverglass (C) and inverted over the depression (D). The coverglass is sealed to the depression slide with paraffin (E).

PLATE I

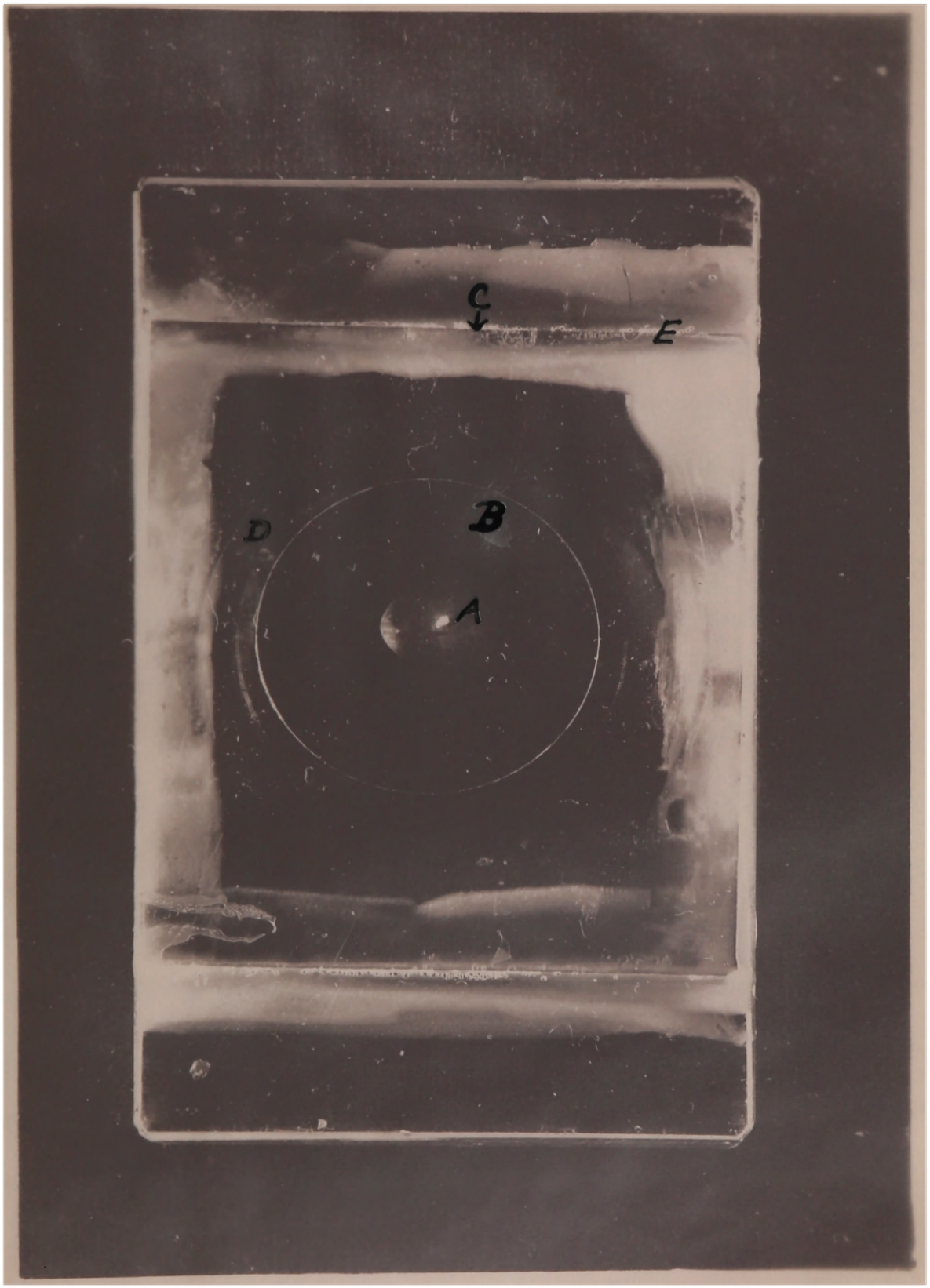
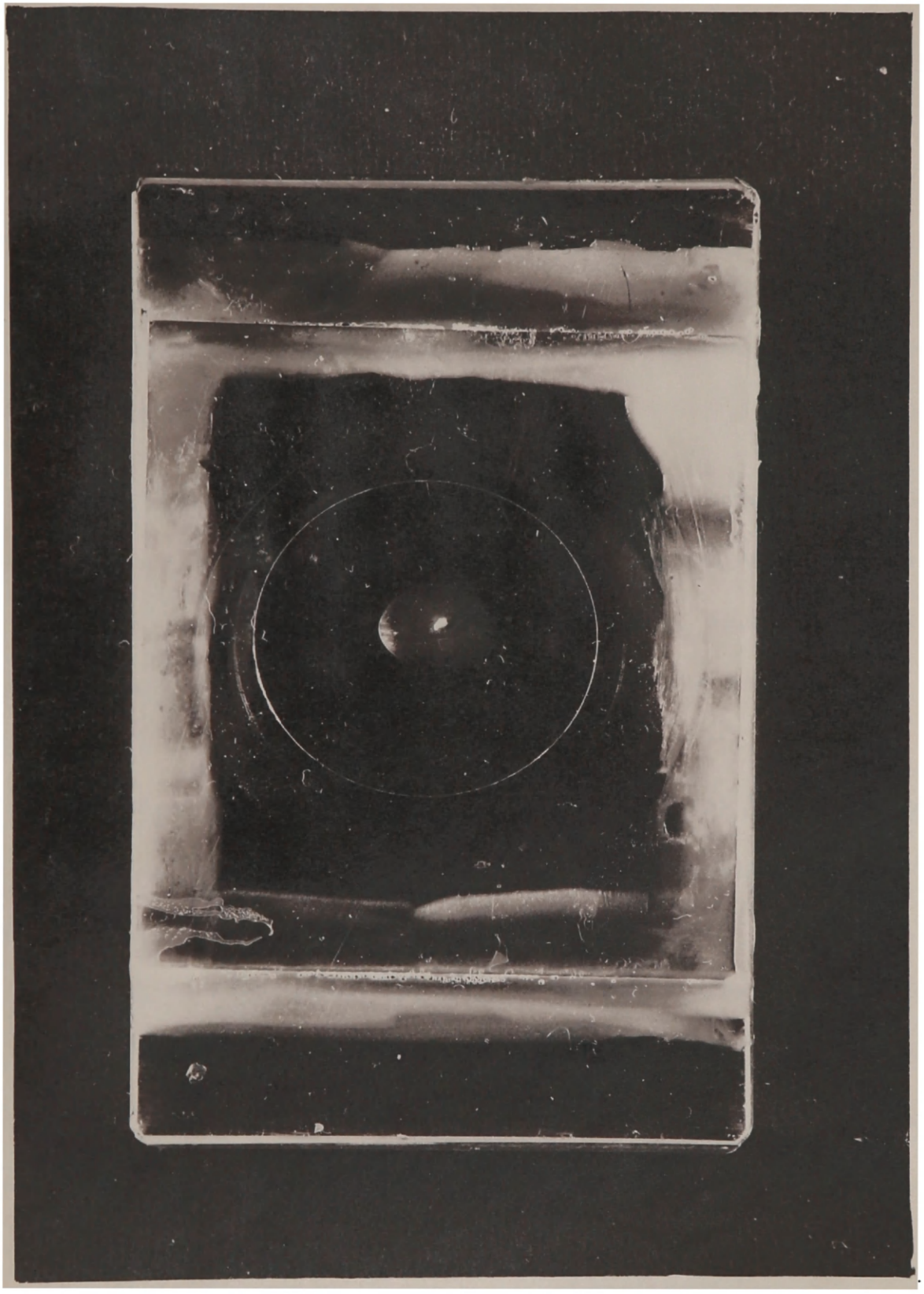


Plate I. Double coverslip culture. Mag. 2x

The explant (A) is on the round coverslip (B) which is attached to the large coverglass (C) and inverted over the depression (D). The coverglass is sealed to the depression slide with paraffin (E).

PLATE I



added if too much of the clot was washed away. A depression slide was placed over the culture and sealed as previously described.

Methods of Study

It was necessary to employ several techniques in order to obtain the desired information from this investigation. The following were the methods used:

Cytological studies. All cultures were examined daily under the ordinary light microscope. The rate of growth of the tissues was noted as well as the presence or absence of bacteria. Cell migration was recorded and the distribution of the bacteria was studied.

Supravital stains, Neutral Red (1:10,000) and Janus Green (1:20,000) were used to identify the type of wandering cells seen at the margins of the explants.

Cultures were fixed and stained in toto in the following manner. The paraffin seal was loosened from the depression slide with a scalpel blade. The large cover glass was carefully rinsed and the small, round coverslip, with the culture adhering to it was floated onto the surface of Bouin's fixative. After approximately one-half hour, the tissue was gradually hydrated through distilled water. With the

tissue still on the coverslip, the entire section was stained in a 20 percent aqueous solution of Harris' hematoxylin for 12 hours. The cultures were gradually dehydrated, stained lightly with eosin, cleared in xylene, and mounted with Permount.* Care was taken to dehydrate the clot completely and clear gradually with xylene before mounting on the slide.

To study the histopathology some of the cultures were fixed in Bouin's for approximately one-half hour. The cultures, while on the coverslip, were dehydrated in ascending strengths of alcohol. In the absolute alcohol, the cultures were shaved off the coverslip with a sharp razor blade. The cultures were passed through several changes of oil of wintergreen, infiltrated with paraffin, and embedded. Serial sections of each block were cut at four microns. Some of the sections were stained with hematoxylin-eosin for histopathological examinations. In an attempt to identify L. monocytogenes in sections, both Gram Weigert and Goodpasture's stain were used (Mallory, 1942). Weigert-Van Gieson stain was employed to study the various types of connective tissues which were involved in the lesions produced.

---

* Permount, Fisher Scientific Co., New York, New York.

Bacteriological studies. In order to test for viability of L. monocytogenes the explants were cultured on either tryptose agar or in broth. After opening the preparations, the entire clot with the explant, was removed with a bacteriological loop and placed on the appropriate media. These cultures were incubated at 37°C. Some tissue cultures were refrigerated for several days prior to checking for the bacteria.

Photography. Photomicrographs were taken with a Bausch and Lomb Model L camera. Photographs of colonies of L. monocytogenes were taken with an Eastman Kodak View camera using a micro-sumar lens.

RESULTS AND DISCUSSION

Bacteriological Studies

Effects of fluids. Before it was possible to do tissue culture experiments, the reaction of the constituents of the tissue culture medium on L. monocytogenes had to be determined.

Medium 199, the nutrient solution, proved to be in no way inhibitory to the growth of the organism. A culture of the bacteria in medium 199 remained viable for more than two weeks. Each day 0.2 ml of the culture was plated onto tryptose agar and the growth always was luxurious after 18 hours of incubation.

When L. monocytogenes was incubated in a plasma clot with medium 199, the bacteria remained viable for more than eight days. As a result of the foregoing experiments it was evident that none of the tissue culture fluids were bactericidal to this organism.

Refrigeration studies. Gray et al. (1948) reported that maceration of tissues followed by refrigeration increased the probability of recovering the organism from tissues; other workers have reported similar results. Osebold and Inouye (1955, 1955a) found an increase of 47 percent in recovery of L. monocytogenes from rabbit tissue

and 95 percent increase in recovery from tissues of sheep. This is a curious phenomenon which remains unsolved at this time.

Because of this strange behavior, inoculated tissue cultures were studied for this same phenomenon. After incubating for 24 hours, the tissue cultures were observed under the microscope for cell migration and the presence of bacteria. The growth of tissues appeared to parallel that of uninoculated controls but microorganisms were seen moving freely about the margins of the infected tissue cultures. In all observations of living cultures, the bacteria were always motile. This will be discussed in greater detail in the section concerned with the description of the living cultures.

When a 24-hour culture was unsealed, and the clot and explant placed in tryptose broth, L. monocytogenes grew luxuriously after 18 hours' incubation. However, if this same procedure was repeated, using a 48-hour inoculated tissue culture, no bacterial growth appeared in the broth after 3 days' incubation even though the bacteria were seen moving in the living tissue culture. This procedure was repeated with a 3-day-old tissue culture with identical results. Beginning of the fourth day of incubation, one tissue culture was removed from the incubator each day for four consecutive days and placed in the refrigerator at 4°C. Sealed cultures were removed

from the refrigerator after 7, 16, 23, and 31 days. The cultures were unsealed and the entire clot and explant were scraped from the round coverslip and placed in tryptose broth and incubated. All these cultures were positive for L. monocytogenes after two days' incubation. The organism was identified by plating broth cultures on tryptose agar and observing the colonies under a binocular dissection microscope employing oblique lighting. L. monocytogenes appear as small, discrete, blue-green colonies. This method of identification has been used very successfully by Gray et al. (1948).

When explants were smeared directly on tryptose agar, omitting the broth, the organism often was seen growing around the small piece of spleen. Plate II illustrates this and shows the characteristic colonial morphology and color of L. monocytogenes.

It is known that under certain conditions this infection with L. monocytogenes is somewhat self-limiting. Rabbits exposed by ocular instillation often recovered and the only symptom of the disease was the intense conjunctival reaction and a slight monocytosis. Sheep and cows suffering from brain infections with L. monocytogenes occasionally recovered, even though they were left with permanent brain damage. Recent experiments by Gray et al. (1955, 1955a) indicate that this organism can remain viable in the uterus of rabbits for

Plate II. A piece of spleen from an infected culture smeared on an agar plate and incubated 48 hours. The bacterial colony can be seen growing out from under the tissue. Mag. 24x

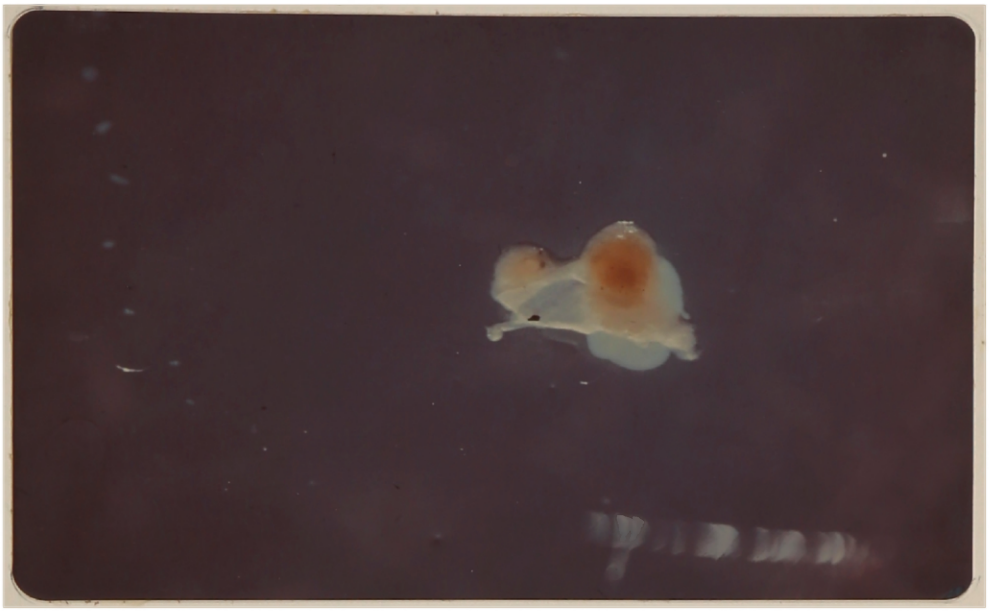
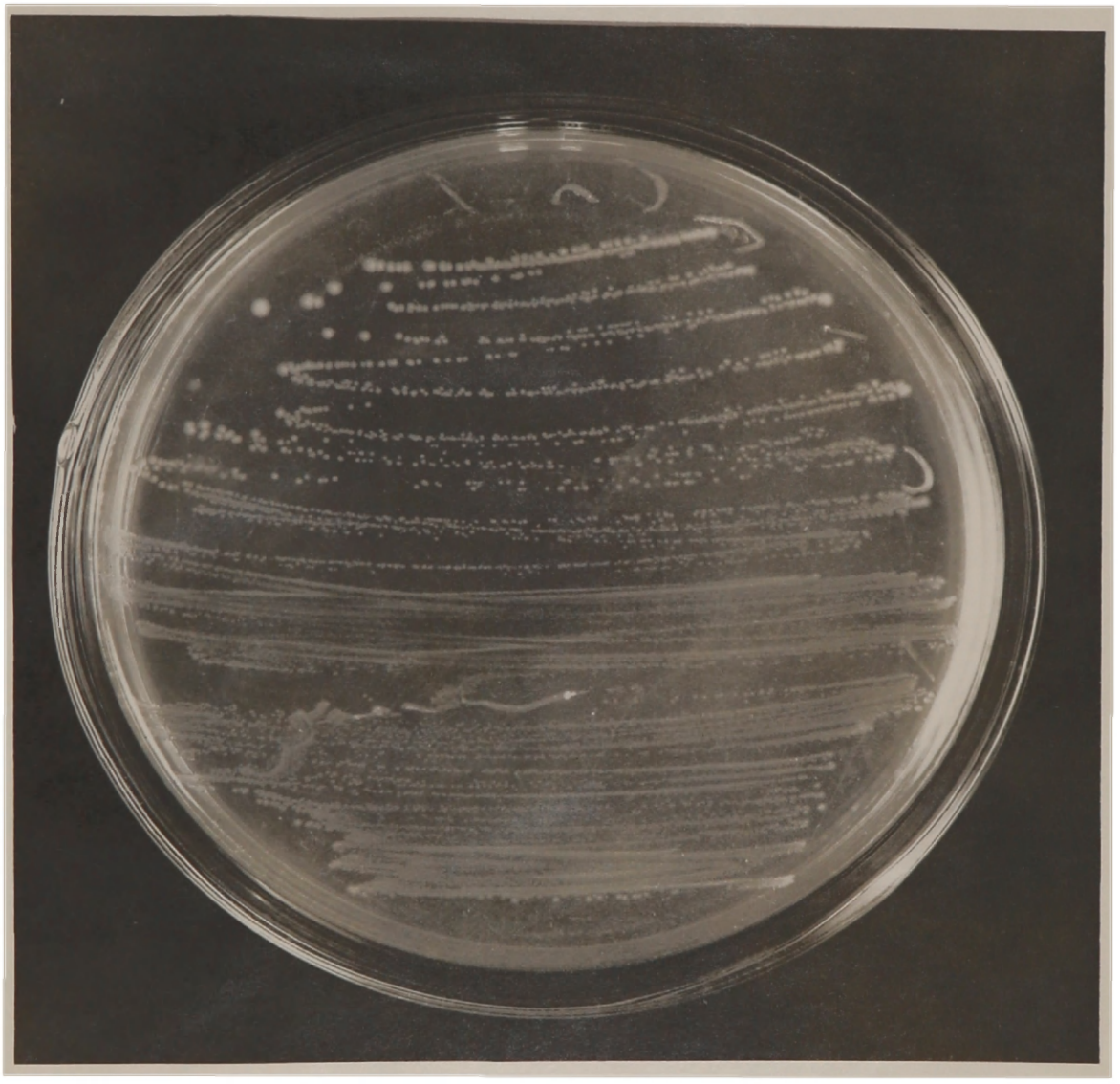


Plate III. Typical L. monocytogenes colonies on an agar plate. Isolated from a tissue culture. Mag. 1.25x

PLATE III



many weeks. When the animals were exposed either orally or ocularly during gestation, abortion occurred; after apparent recovery of the doe, successive rebreeding always terminated in abortion. In every instance, L. monocytogenes was isolated from aborted fetuses and cotyledons. The does did not manifest clinical symptoms of illness and usually survived. However, occasionally after relatively long periods of survival, the rabbits became ill and died. The organism in such cases was isolated from most of the viscera.

Is it possible to correlate the phenomenon of increasing recovery of L. monocytogenes following refrigeration with the fact that the organism can remain in the animal without displaying symptoms? Perhaps some inhibitory substance acts as a bacteriostatic agent at 37°C, but when refrigerated no longer retains this bacteriostatic property. All this is simple speculation without proof at the present time. Nevertheless, in view of the fact that inoculated tissue cultures behaved after refrigeration the same as other tissues treated similarly suggests that some sort of biological system which is inactivated at 4°C allows the bacteria to grow on artificial media. It must be remembered that in every case, prior to making bacteriological cultures of the explants, living, motile bacteria were seen in all inoculated tissue cultures. Therefore, something made

it impossible to culture the organism on artificial media until they were held at 4°C for several days. The answers to these perplexing questions still remain a mystery to be unfolded.

Description of Living Cultures

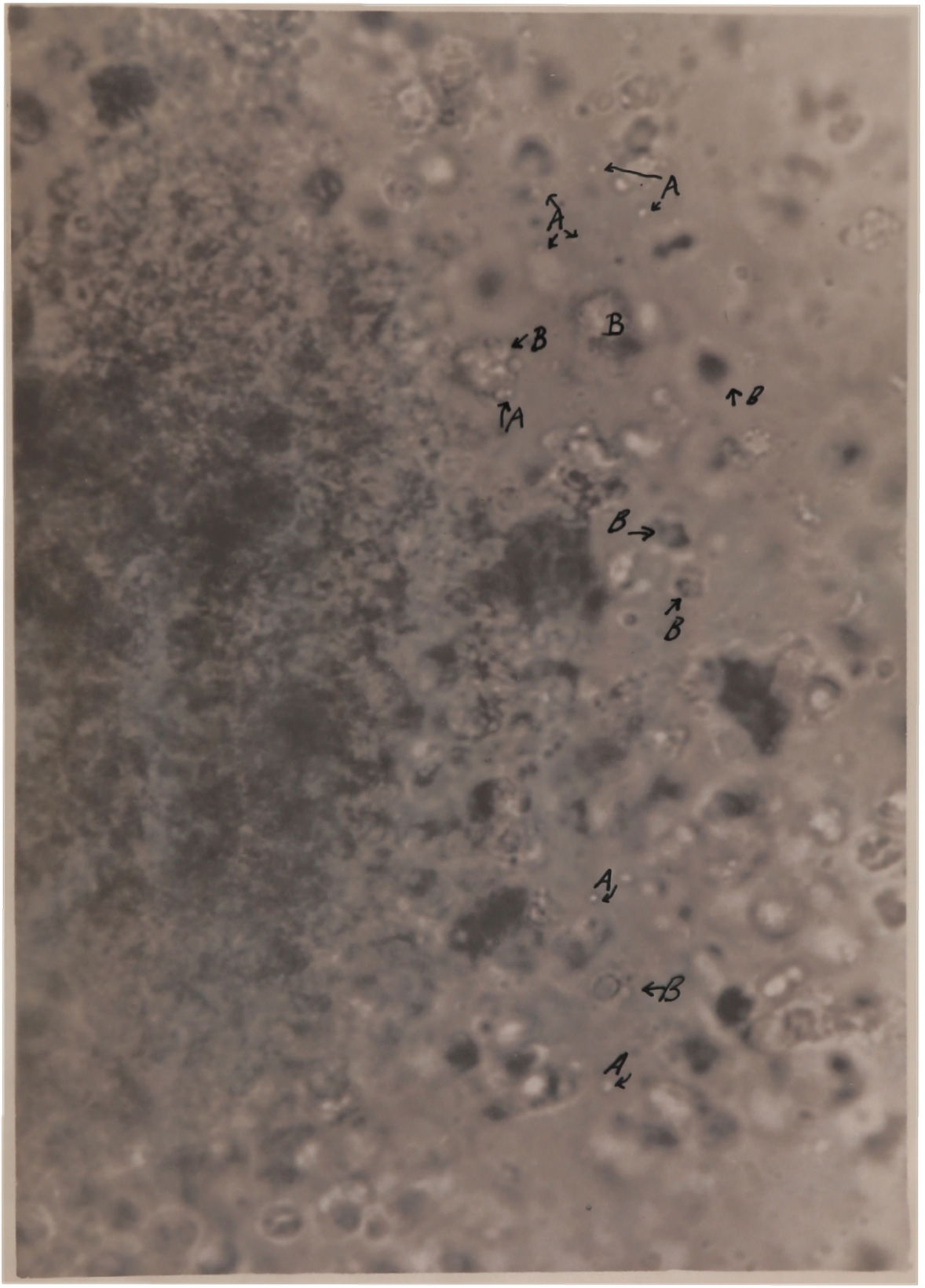
Approximately 290 cultures were grown and observed. Some were fixed and stained in toto, others were blocked and sectioned. Whatever the fate of the cultures, all were observed in the living state.

During the first 24 to 40 hours after incubation, the polymorphonuclear leucocytes migrated outward from the explant, gradually became inactive and degenerated. The neutrophils eventually were obscured by the growth and migration of lymphocytes and monocytes. Plate IV illustrates three days' growth of a splenic culture. Large ameboid cells moved slowly and did not appear to have any particular attraction for the bacteria (Plate V). As previously mentioned, in all observations of inoculated cultures the bacteria were always motile. On several occasions the organisms moved directly toward the macrophages, hammered the cell membrane several times, then backed away as if to gain momentum for another bombardment. This repeated method of attack often continued for

Plate IV. Inoculated living splenic culture three days old. Mag. 200x

Plate V. Inoculated living splenic culture six days old. (A) Bacteria along the margin of the explant. (B) Mononuclear phagocytes. Mag. 940x

PLATE V



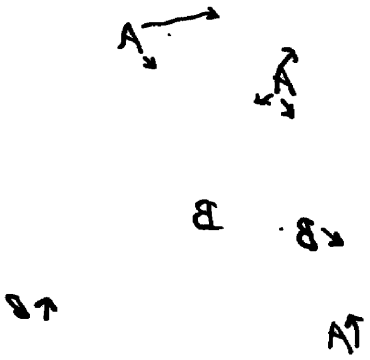
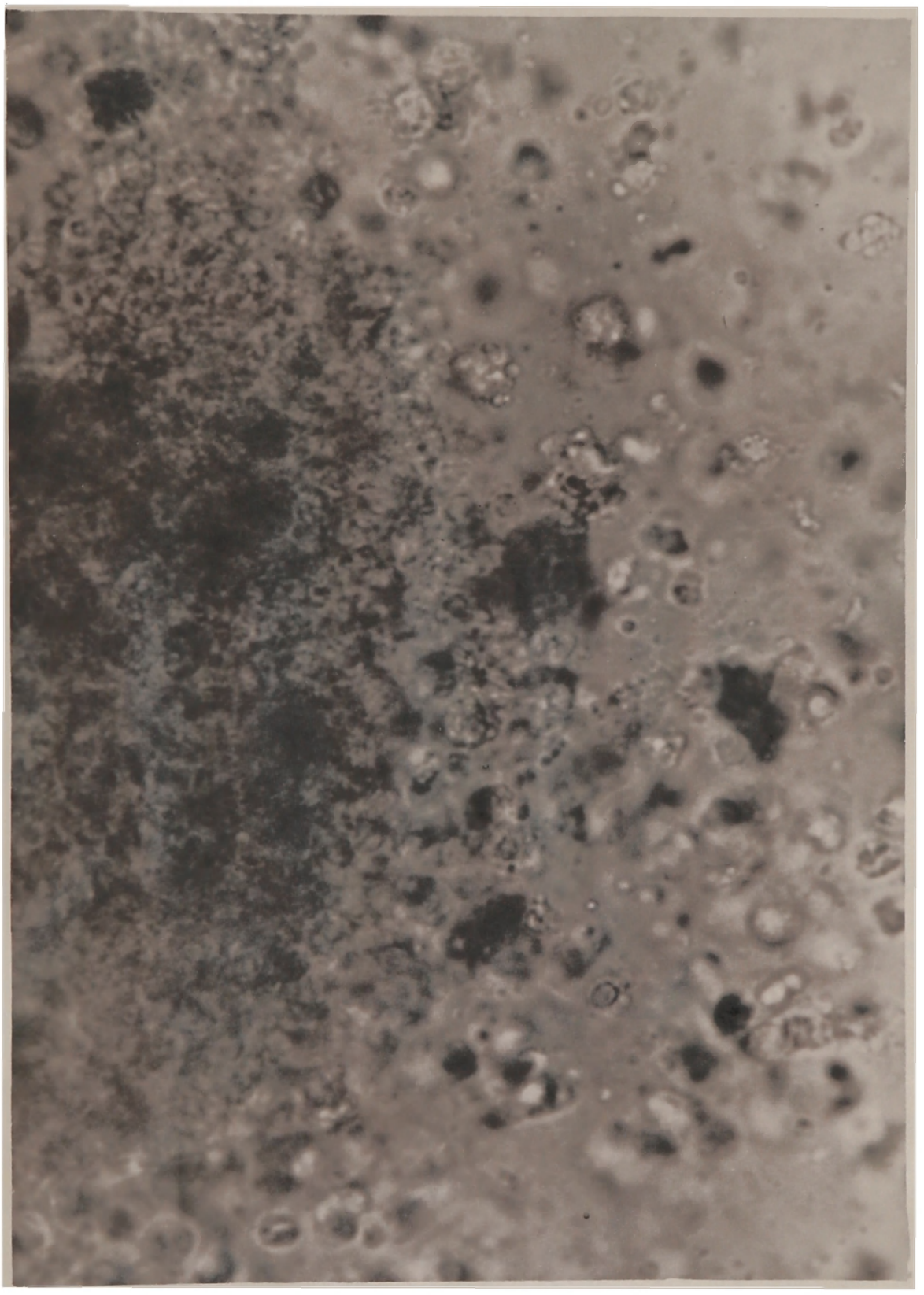


Plate V. Inoculated living splenic culture six days old. **(A) Bac**teria along the margin of the explant. **(B) Mononuclear** phagocytes. Mag. 940x

PLATE V



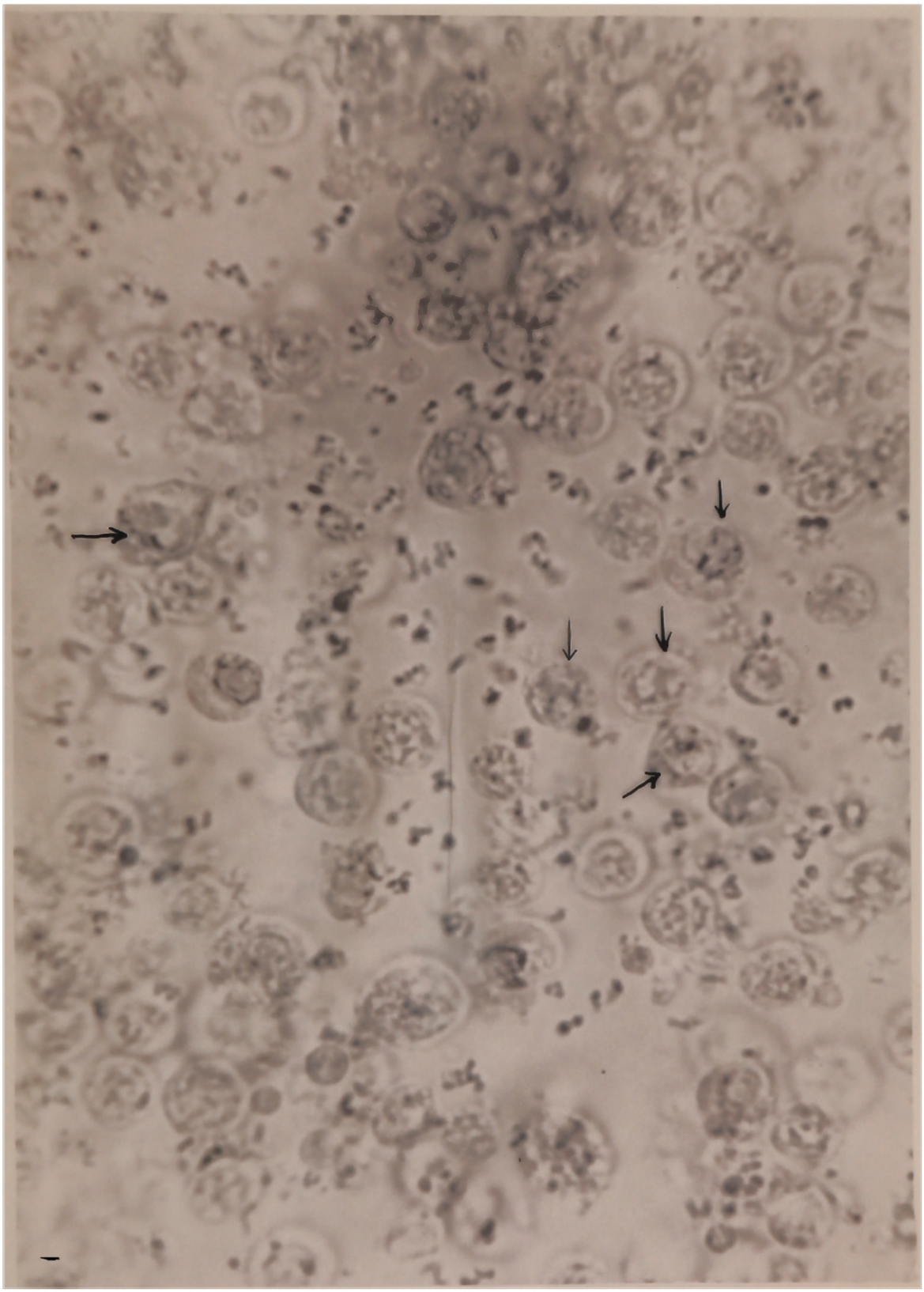
several hours and finally the bacteria entered the cytoplasm of the macrophages. These movements of the bacteria seemed to be purposeful rather than random. Plate VI illustrates the diffuse distribution of the bacteria throughout the culture. This particular culture was stained with Janus Green and the organisms have an affinity for the dye. The bacteria can be seen in several macrophages as well as free in the surrounding medium. Plate VII is an oil immersion photograph of a two-day-old culture. These cells were observed for nearly four hours and during this time several bacteria penetrated the cell membrane.

A great deal of difficulty was encountered in the identification of the cells in the living, unstained cultures. It is sufficient to say that they were mononuclear phagocytes.

Cunningham et al. (1923) identified two distinct types of phagocytic cells in the spleen of the rabbit. One group, consisting of clasmatocytes, macrophages, and histiocytes were said to react intensely to Neutral Red. The nuclei were located peripherally and were relatively small in comparison to cell size. The other group was identified as typical monocytes of the peripheral blood. These were 15μ to 30μ in diameter with a horseshoe-shaped nucleus. When these cells were stained with Neutral Red there was a central, clear

Plate VI. L. monocytogenes stained supravitally with Janus Green in a five-day-old living culture. The bacteria appear in V, Y, and pallisade forms. Macrophages containing bacteria are marked with arrows. Mag. 1500x

PLATE VI



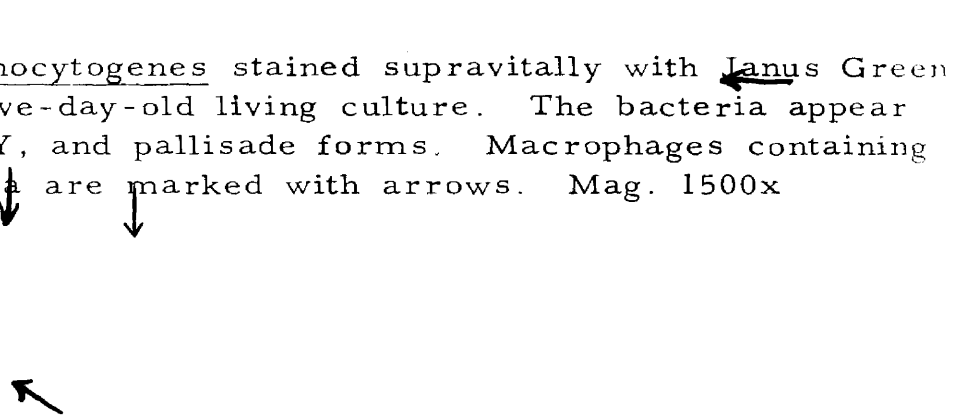


Plate VI. L. monocytogenes stained supravitally with Janus Green in a five-day-old living culture. The bacteria appear in V, Y, and pallisade forms. Macrophages containing bacteria are marked with arrows. Mag. 1500x

PLATE VI

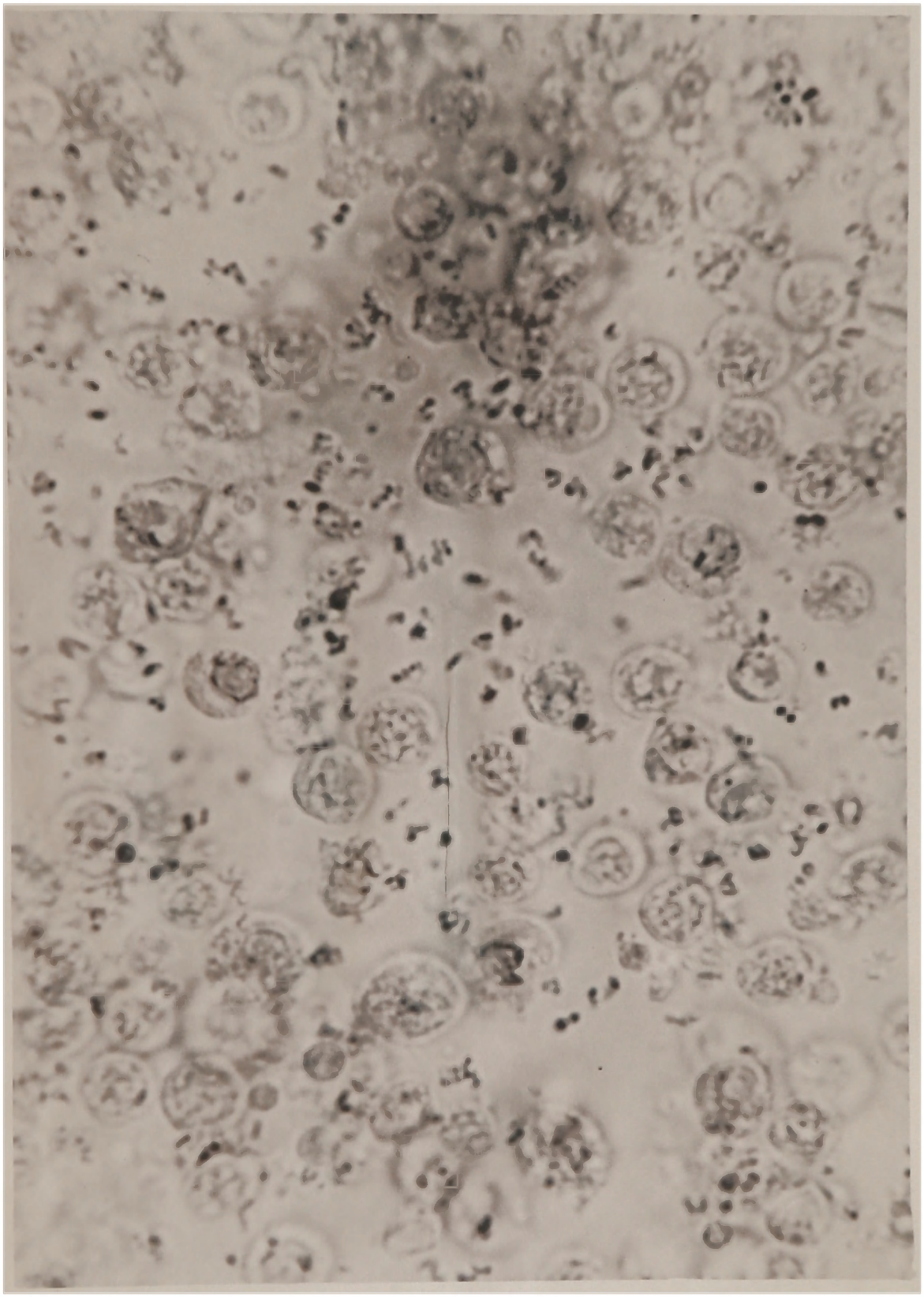
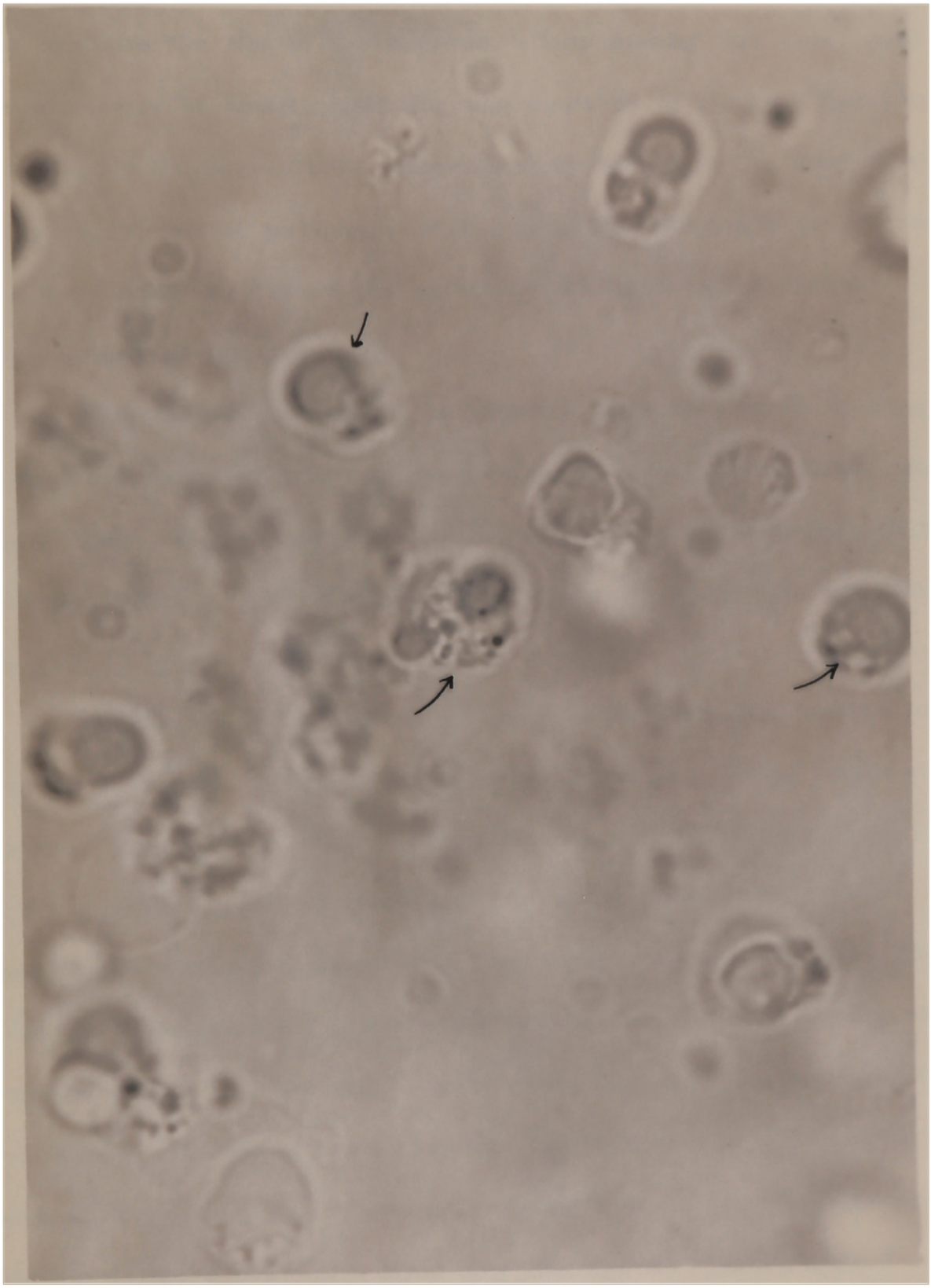


Plate VII. Mononuclear phagocytes in a living two-day-old culture. Bacteria can be seen in those cells marked with arrows. Mag. 3050x

PLATE VII



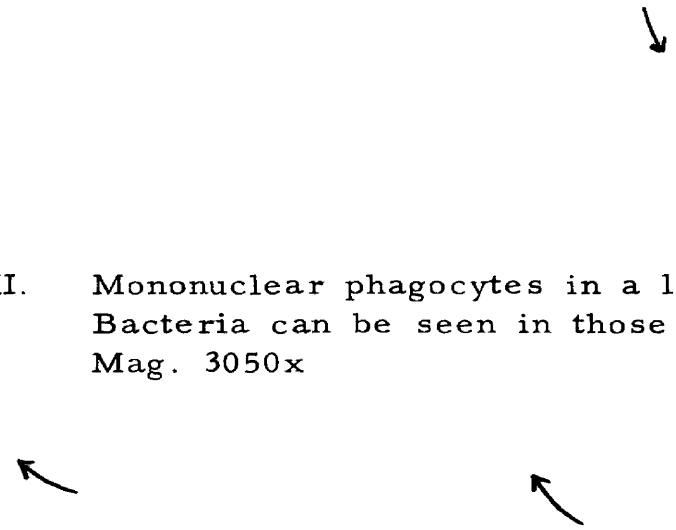
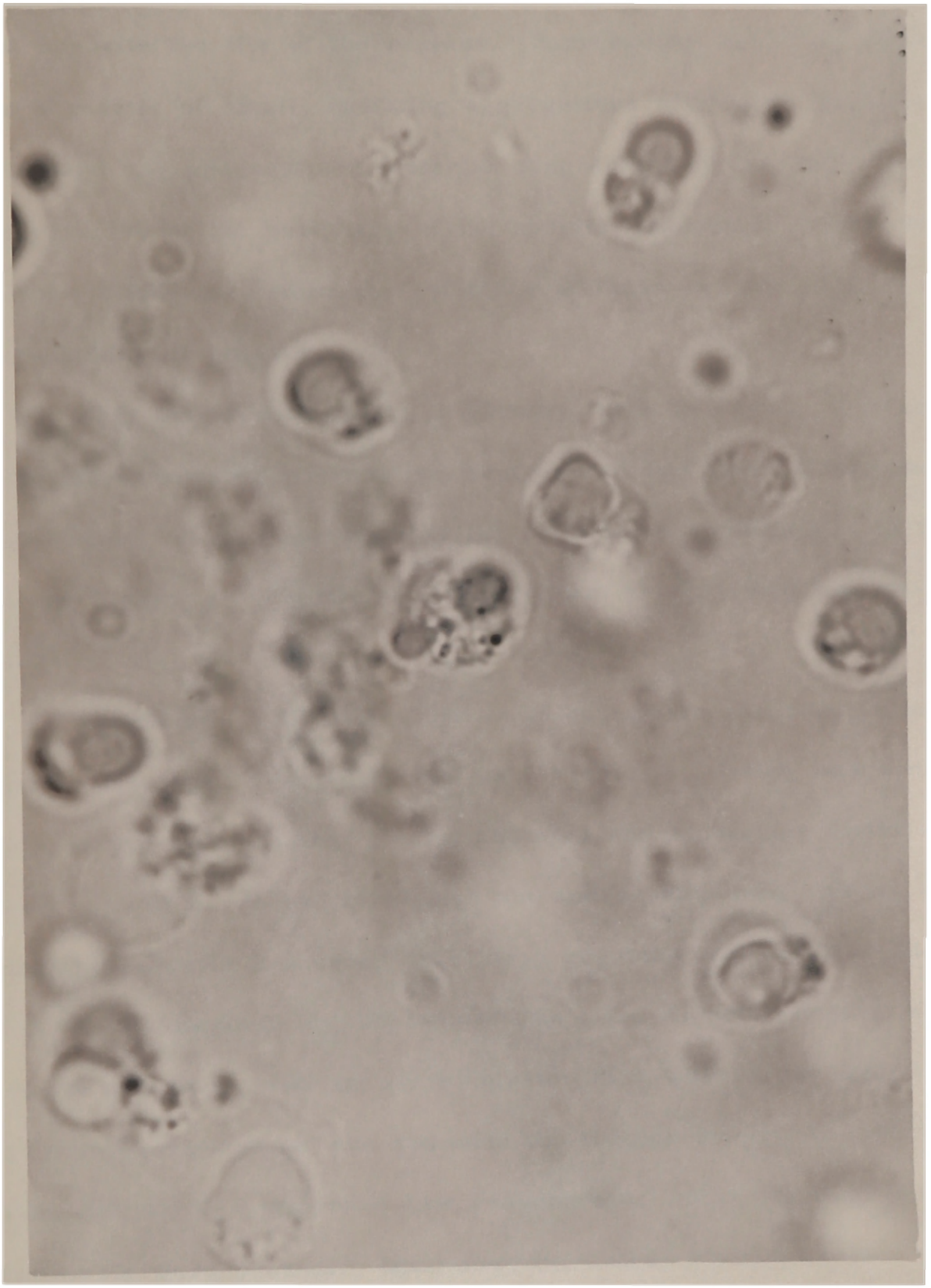


Plate VII. Mononuclear phagocytes in a living two-day-old culture. Bacteria can be seen in those cells marked with arrows. Mag. 3050x



spot opposite the Hof of the nucleus. Surrounding this clear spot was a rosette of small, radiating aggregations of Neutral Red particles and any phagocytized material congregated in the zone of the red granules at the periphery of the rosette. McJunkin (1926) and Forkner (1930) confirmed the existence of these two varieties of mononuclear phagocytes.

This author was unable to identify these two types of phagocytes after repeated trials using Neutral Red and Janus Green supravital stains. It was obvious that the cells were mononuclear in type but the rosette as described by Cunningham et al. (1923) was never seen.

Maximow (1927) was of the opinion that the nongranular leucocytes developed into polyblasts and fibroblasts. Using tissue culture methods, he reported that the lymphocytes and monocytes at the end of the second day began to stretch out and develop long, spear-shaped, nonmotile processes with large regular, oval nuclei. These cells were described as "fibroblast-like" and as soon as they were brought into focus with an oil immersion lens with strong light they contracted, rounded off and resumed the former ameboid wandering cells with motile membrane-like pseudopodia. After six days typical fibroblasts were present. Maximow, therefore, reasoned that

lymphocytes developed into polyblasts then into fibroblasts. This theory seems rather presumptuous, but there is no proof that it is wrong. The origin and development of mononuclear macrophages still remains a very controversial subject and it is not the purpose of this writer to take a stand on this matter. It can only be stated that the cells identified in the living cultures were mononuclear phagocytes.

When the tissue culture experiments were first started, it was difficult to determine how many bacteria to add to the explanted tissue. Pilot experiments in which thirty organisms per milliliter were used proved this to be an inadequate dose. In previous animal experiments by Gray (1954), the concentration of bacteria obtained from a suspension made to match the 0.5 tube of a McFarland nephelometer was found to be an adequate dose to produce symptoms and lesions typical of L. monocytogenes infection. With this information as a basis, the same suspension was used in the tissue cultures. The suspension contained 2.289×10^9 organisms per milliliter; therefore, in 0.01 ml there were 2.289×10^7 bacteria. This concentration of bacteria did not suppress the growth of the tissue (Plate V). It is interesting in view of the reports by Smyth (1915, 1916) and Lewis (1920) which stated that the tissue did not remain alive in

the presence of bacteria. Tissue cultures inoculated with L. monocytogenes never demonstrated growth suppression or inhibition. Plates VIII and IX illustrate profuse growth of cells after six and seven days of culture, respectively. Plate X is an illustration of a six-day uninoculated culture.

Perhaps the early workers had difficulty in maintaining tissue cultures inoculated with bacteria because of inferior nutrient fluids. There is little doubt but that artificial media similar to 199 exert tremendous influence upon the viability of tissues in vitro. The pioneers in this field were less fortunate in having to rely on simple salt solutions to which they added serum and embryo extract.

Description of Fixed Cultures

Whole mounts. Cultures which were fixed in toto were stained with hematoxylin-eosin or Wright's stain.

Many polymorphonuclear cells were prominent during the first 24 to 36 hours of culture. These cells were rather diffusely distributed and some contained engulfed organisms.

After 48 hours the majority of the cells were macrophages. Many of the cells exhibited bizarre forms, such as club, dumbbell, hook, and spindle shapes. The cells were most numerous at the

Plate VIII. A six-day-old living culture showing good growth of tissue even though infected with bacteria. Mag. 200x

PLATE VIII

Plate IX. A seven-day-old living culture showing heavy growth of fibroblasts. The presence of bacteria did not inhibit the growth of the tissue. Mag. 75x

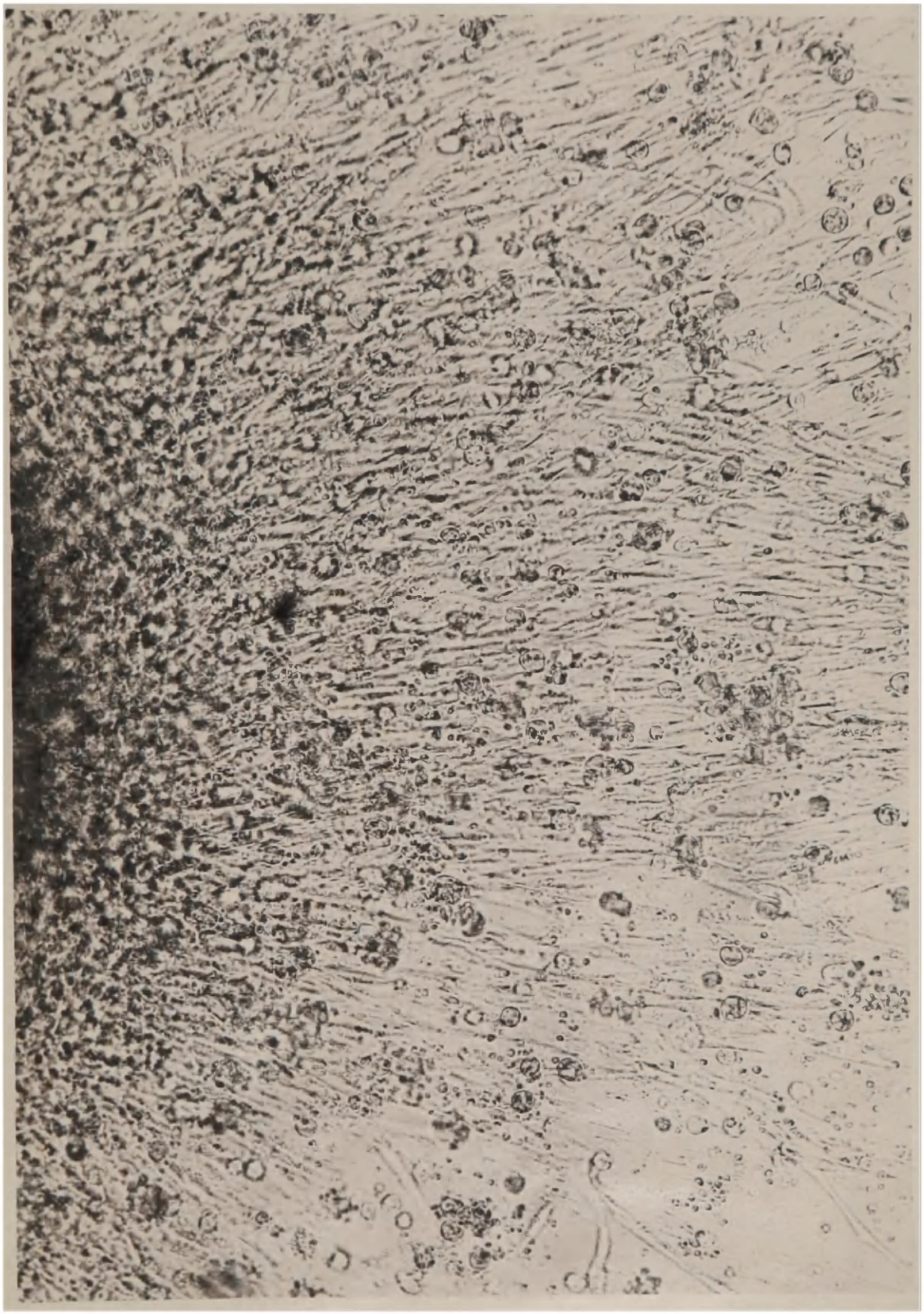
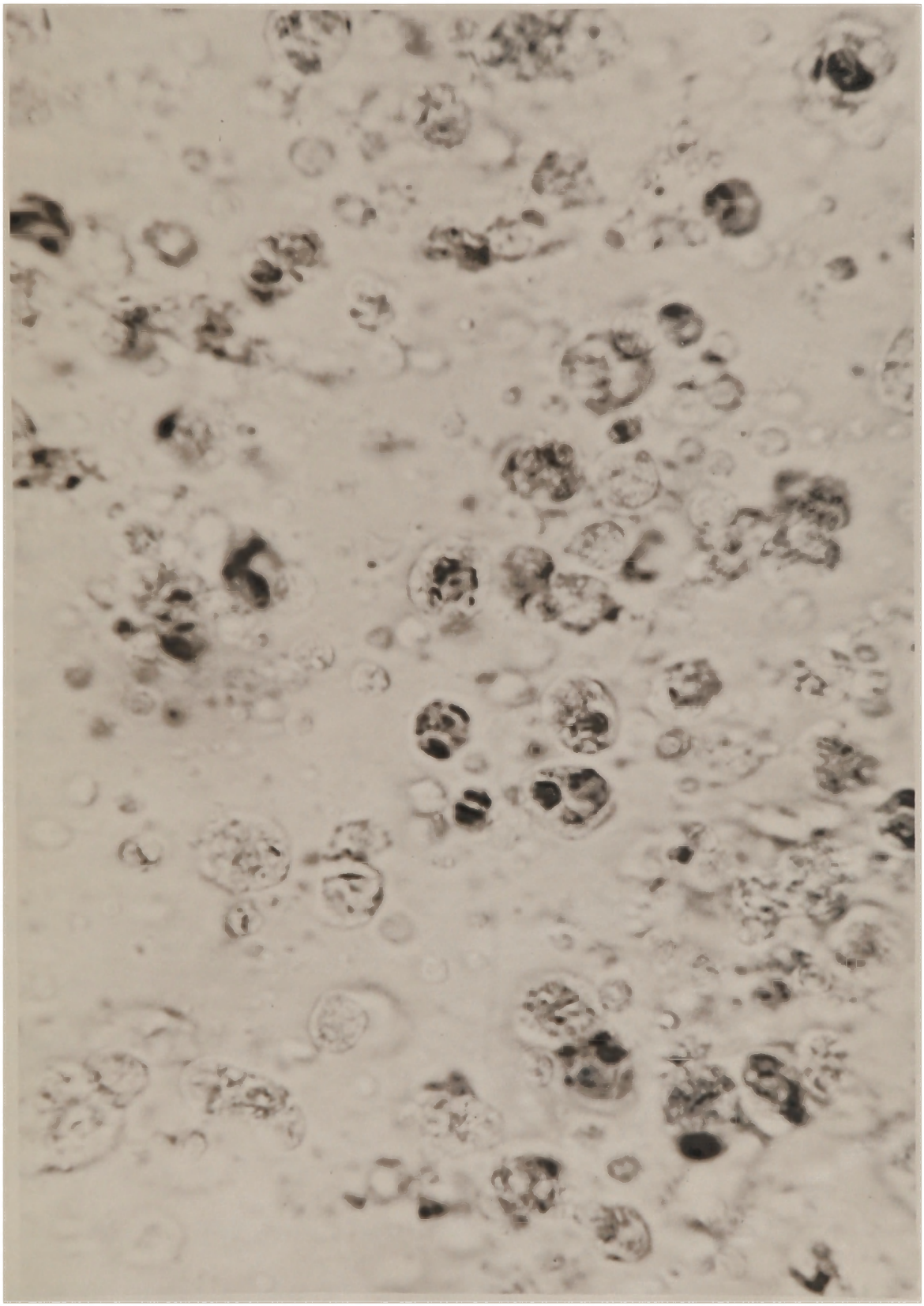


Plate X. An uninoculated control culture. Six days old. Mononuclear phagocytes are the predominating cells. Mag. 1600x

PLATE X



margin of the explant. A small amount of cytoplasm was seen at each end of the cells. Plate XI, Figures 1 and 2, show examples of these cells. These bizarre cells were seen in cultures as old as four days; however, they were not present at all in uninoculated controls.

Hagemann and Simon (1953) and Hagemann et al. (1953) described bizarre nuclear forms in granulomas. In their opinion the cells arose as a result of hindered proliferation of capillary wall tissue due to the stimulus of a toxic reagent. The aberrant cellular forms observed in the inoculated tissue cultures were similar to those described by Hagemann and Simon (1953) and Hagemann et al. (1953). Most of these cells with bizarre nuclei were found in the area immediately adjacent to the explant.

After three or four days the bizarre forms were not as prominent and after six days they were only rarely seen. The cells most often seen at this stage were round cells with vesicular nuclei as pictured in Plate XII. Most of these were at the margin of the tissue, while out in the surrounding media, plasma cells and lymphocytes were most prominent.

More study with stained whole mounts would be desirable. However, these preparations present some difficulty in handling.

Plate XI. Bizarre nuclear forms seen in 48-hour cultures. Stained with hematoxylin-eosin. Mag. 1650x

PLATE XI

Figure 1

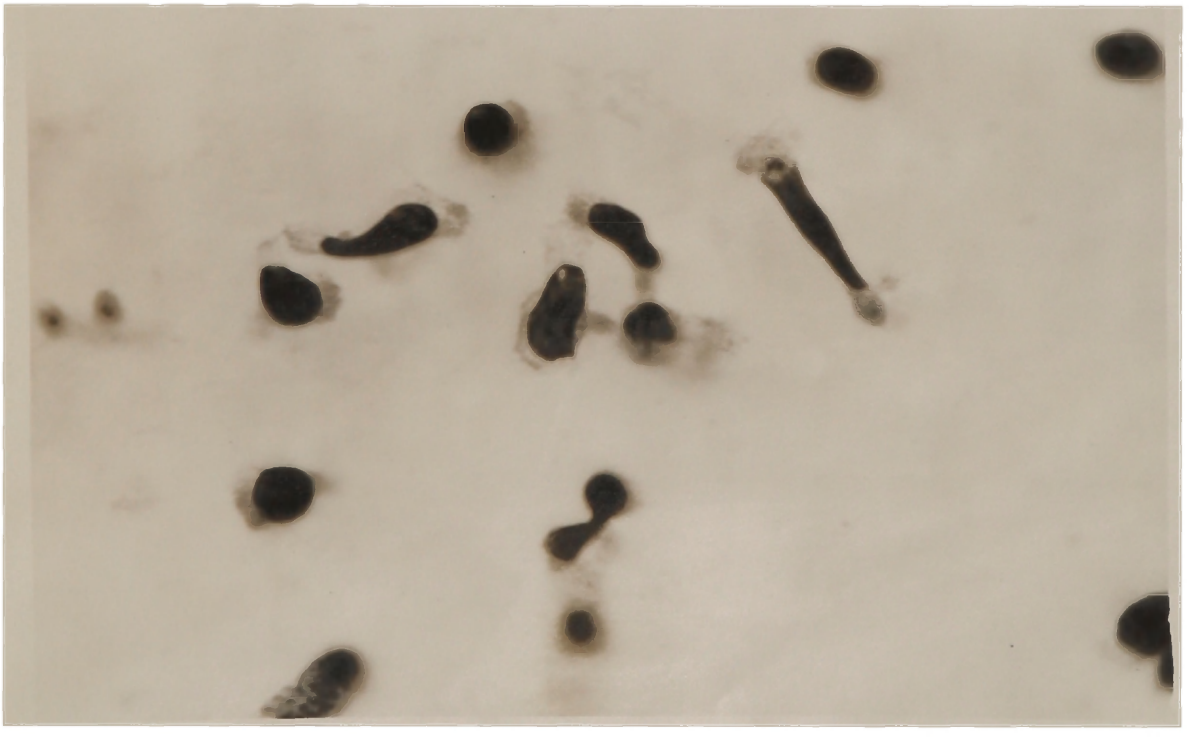
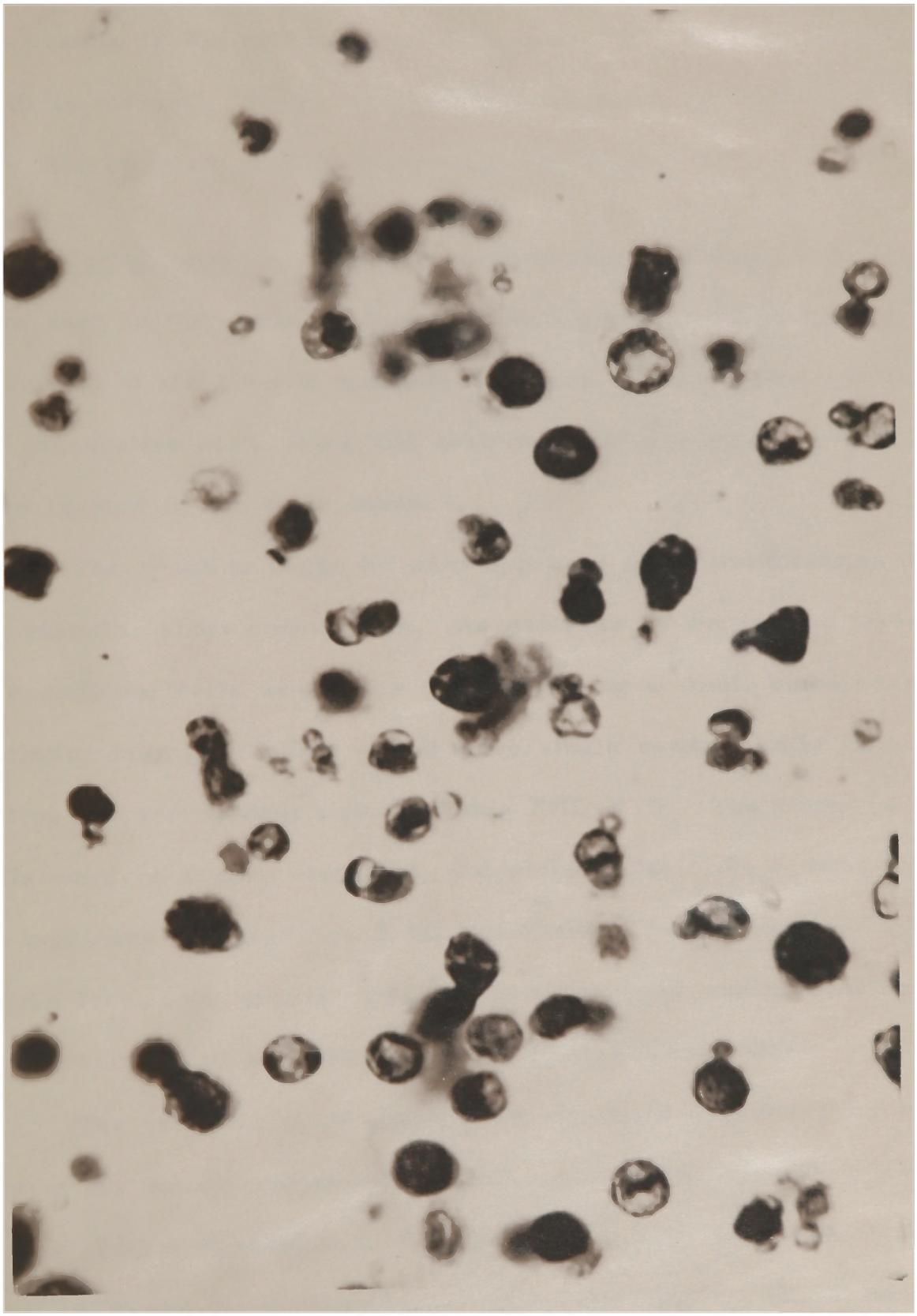


Figure 2

Plate XII. Cells with vesicular nuclei seen in three-day-old cultures. Stained with hematoxylin-eosin. Mag. 1860x

PLATE XII



The protein in the clot tends to absorb the stain and much cytological detail is masked. Better preparation may be possible by the use of very thin clots or perhaps perforated cellophane.

Serial sections. The most outstanding pathological changes were seen in the serial sections of the explants. Tissue cultures, inoculated at the time of explantation, were removed each day for six consecutive days, fixed and sectioned. Uninoculated controls were treated in the same manner.

The reaction began by what appeared to be proliferation of the vascular sinus endothelium. As early as 24 hours, two layers of endothelial cells were seen circumscribing a small blood vessel. Radiating from this blood vessel were single rows of cells infiltrating the surrounding tissue (Plates XIII, XVI). The nuclei of these cells were vesicular, elongated, and pleomorphic. As a result of the continued proliferation of the endothelium, the cells appeared as swirls around the vessels until the involved area was of considerable size at the end of the six-day period (Plates XV to XXVI).

The vascular sinus endothelium has been considered as a part of the reticuloendothelial system. One group of cells belonging to this system is composed of large mononuclear wandering cells. They are found in all tissues and circulating fluids. The designations

of the cells change with respect to their location. In the tissues they are usually referred to as tissue macrophages, histiocytes, or clasmatocytes, while in the circulatory system they are called monocytes. The origin of mononuclear phagocytes has been a subject of considerable controversy. Mallory (1898) and Foot (1925) were of the opinion that the mononuclear cell was derived from the endothelial cell. On the other hand, Maximow (1924a), Lang (1926), and Bloom (1928a) did not believe that the endothelium gave rise to cells possessing ameboid characteristics. Sabin et al. (1925) reported still another theory, stating that the clasmatocytes were derived from the endothelium and the monocytes from "reticular" cells. Lewis (1925), while not in agreement with Sabin et al. (1925), favored Maximow (1924a) to some extent, and also believed a part of Mallory's theory (1898). Today, some thirty years later, the question remains unanswered. Forbus (1943) stated that the mononuclear wandering cells were derived either from primitive mesenchymal cells or from vascular sinus endothelium. The latter view seems to be the most widely accepted one today.

Even though the origin is most controversial, the function is clearly phagocytosis. It is well known that these cells show a remarkable capacity for proliferation and pleomorphism. Multinucleated giant cells and epithelioid cells are derived from these cells.

Sections treated with Weigert-Van Gieson connective tissue stain exhibited a single elastic connective tissue fiber around the lumen of the reactive blood vessels (Plate XXIV, Figure 2). The epithelioid cells circumscribed the area forming a solid mass.

Simon (1953) stated that the formation of the granuloma was apparently connected with capillary tissue. Hagemann and Simon (1953) reported that the entire connective tissue of the vessel was involved in the primary proliferation of mesenchymal cells. Hagemann et al. (1953) claimed that the capillary endothelium phagocytized the bacteria and also that the vessel reaction isolated the focus of the infection and set up a granuloma. Therefore, the cellular component of the granuloma was thought to be vascular endothelium.

The careful day-by-day studies of the tissue culture sections revealed a similar reaction. It was especially obvious when 24 or 48 hour cultures were studied. At this time only one or two layers of cells surrounded the blood vessels, with a few cells trailing off into the nearby tissue. Another interesting observation revealed that this characteristic reaction never was seen except in areas in which blood vessels were present. This seemed to indicate that these cells have some intimate association with the vascular endothelium.

At the margin of the tissue cultures at least four days old, exceptionally large, round macrophages were seen (Plate XXIII). They appeared to be the same cells as those in the tissue but were more spherical and usually contained a golden yellow pigment. Maximow (1924), in his work with the tubercle bacilli in tissue culture, reported a similar finding. He was of the opinion that this material was digested bacteria. However, in the present study repeated attempts were made with special stains, but L. monocytogenes was never demonstrated in the peripheral cells or in the reactionary cells of the explant proper. As previously mentioned, tissue cultures infected with L. monocytogenes required refrigeration in order to demonstrate the organisms in artificial culture medium. Hagemann and Simon (1953) reported difficulty in finding bacteria in the granulomas, but were able to culture the organism from these structures. As a matter of fact, the granuloma was often called a "Listerioma" by these investigators because they were of the opinion that this structure was specific for the disease, granulomatosis infantiseptica.

In the center of the explants some necrosis was usually present after 48 to 72 hours of cultivation; this was to be expected simply from the mechanical aspect. The center of the tissue did not receive the benefit of the nutrient fluid. The necrosis appeared in the controls as well as in the inoculated cultures.

The endothelial reaction usually occurred at the periphery of the tissue and often long rows of the proliferating cells infiltrated the area of necrotic tissue as if to digest the cellular debris. A similar reaction was reported by Hagemann and Simon (1953) and Hagemann et al. (1953) in describing the histopathology of granulomatosis infantiseptica. They described a centripetal infiltration of "the capillary cells of resorption" which engulfed the entire focus forming a primary "proliferating granuloma." These granulomas were found in the organs of stillborn infants from which L. monocytogenes was isolated.

Most of the American workers who have described the histopathology associated with L. monocytogenes infections, singled out necrosis as being the primary lesion. However, Julianelle and Moore (1942) reported finding many macrophages surrounding the necrotic area and drew attention to the fact that this was a peculiar finding to be associated with a necrotic reaction. Other workers have reported that fibroblasts were present; this makes one wonder if they were really fibroblasts or if they were actually proliferated cells of the reticuloendothelial system. The nuclei of young fibroblasts often resemble immature mononuclear cells.

As previously mentioned, Hagemann and Simon (1953) believed that there are two distinct cellular reactions possible resulting from

infection with L. monocytogenes. One was characterized by a granulomatous inflammation, the other by necrosis. They also reported that a combination of these two reactions can occur within the same area. These facts may help to explain some of the confusion existing regarding the histopathogenesis of this disease.

According to Forbus (1949) a pure granulomatous inflammation is an "exudative" and "productive" reaction in the tissues characterized by the accumulation and proliferation of reticuloendothelial cells from the circulating fluids and the tissue spaces. The predominant cell in the reactive area is the macrophage in contrast to other inflammatory processes in which neutrophils or lymphocytes are involved. Forbus (1949) stated that this inflammatory response can be acute or chronic.

Several bacteria have been associated with a granulomatous reaction, such as Brucella suis, Pasteurella tularensis, Mycobacterium tuberculosis and Mycobacterium paratuberculosis. It has been suggested by some investigators that these organism may exist intracellularly, and there is some evidence that L. monocytogenes is intracellular at times. Since the previously mentioned organisms produce a granulomatous inflammation it may be possible that L. monocytogenes possesses some of the same characteristics. Work

done in Germany supports this theory to a certain extent, and most certainly the reaction produced in the tissues cultures was granulomatous in nature.

The possibility that the response seen in the tissue cultures may be a nonspecific reaction due to an irritant must not be overlooked. Further studies must be made to determine if this lesion produced in vitro is specific for L. monocytogenes.

Plate XIII. A section of explant after 24 hours' incubation. Two rows of proliferated vascular endothelium can be seen. Mag. 400x

PLATE XIII

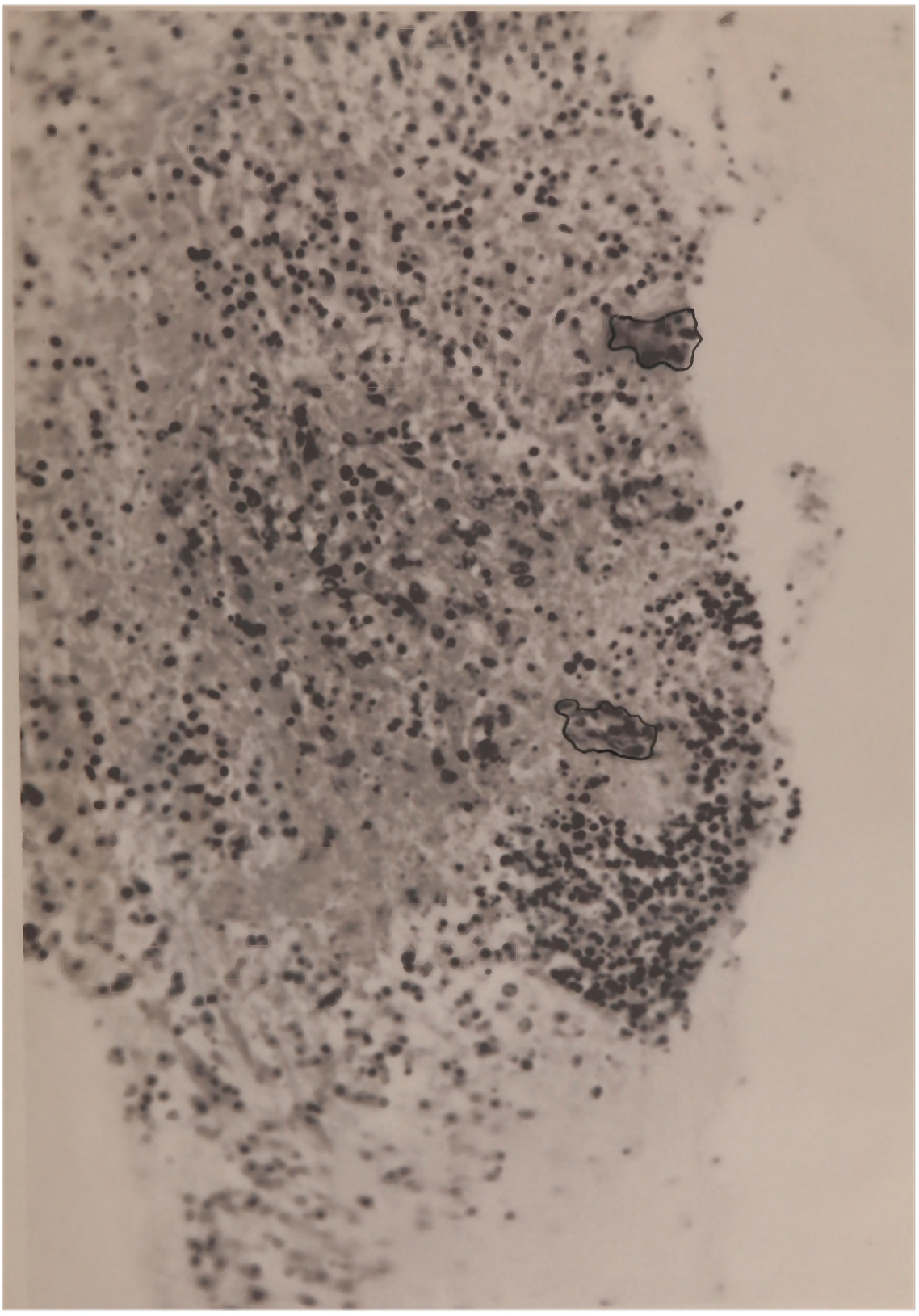


Plate XIII. A section of explant after 24 hours' incubation. Two rows of proliferated vascular endothelium can be seen. Mag. 400x

PLATE XIII

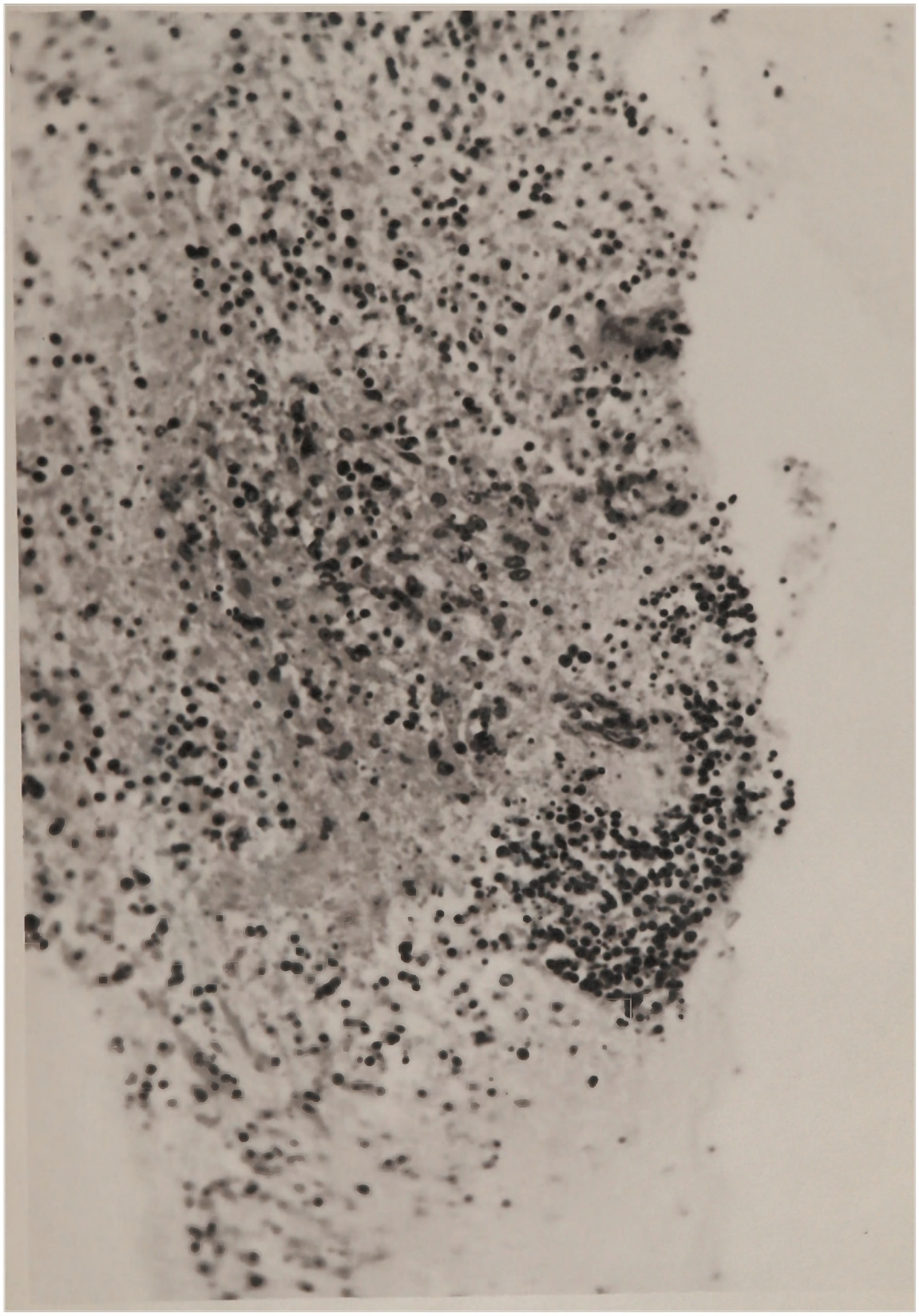


Plate XIV. High power of area marked in Plate XIII. Mag. 1200x

PLATE XIV

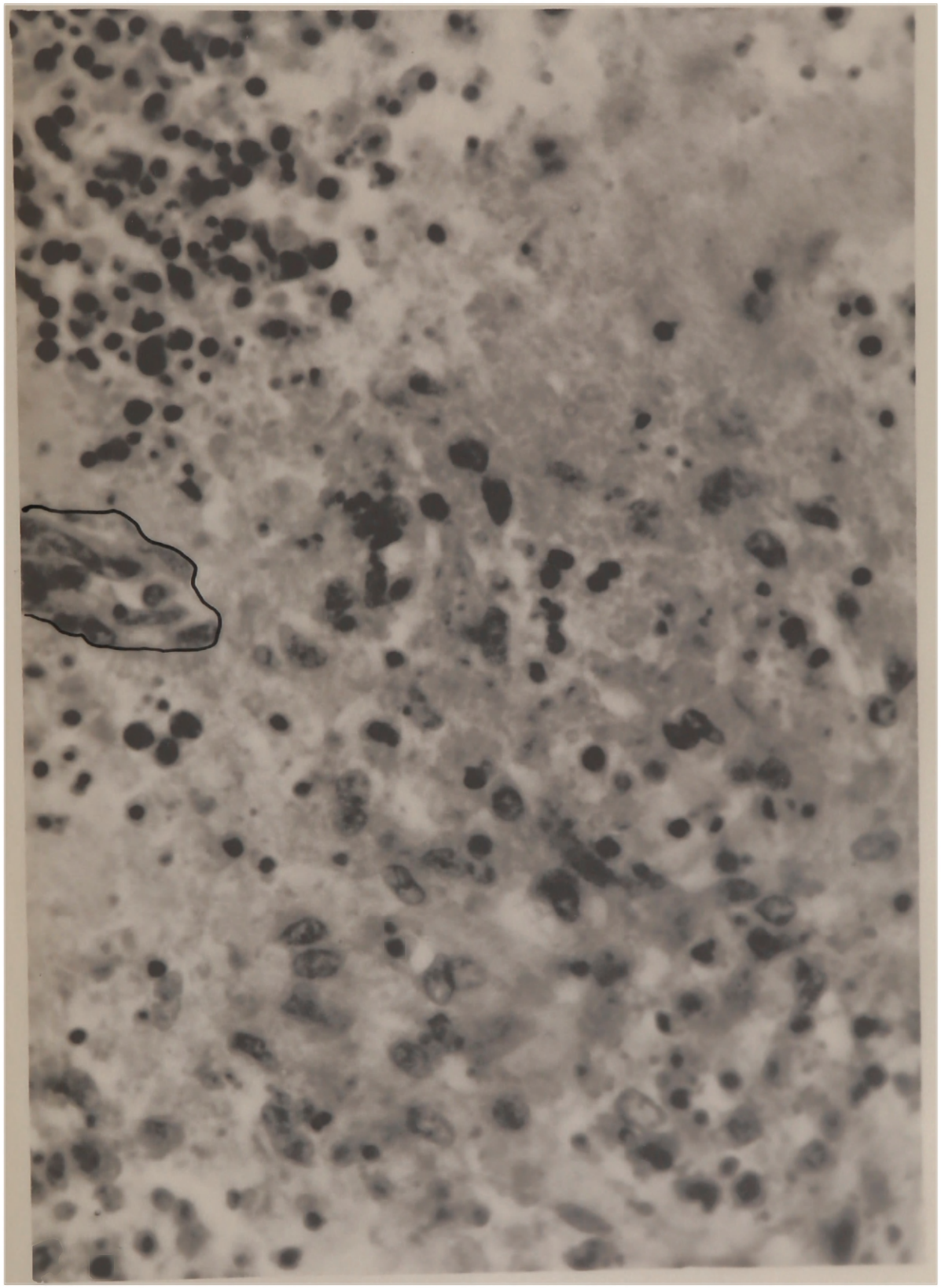


Plate XIV. High power of area marked in Plate XIII. Mag. 1200x

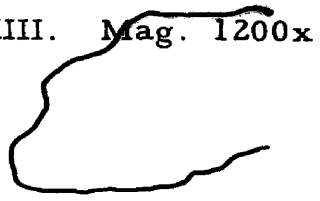


PLATE XIV

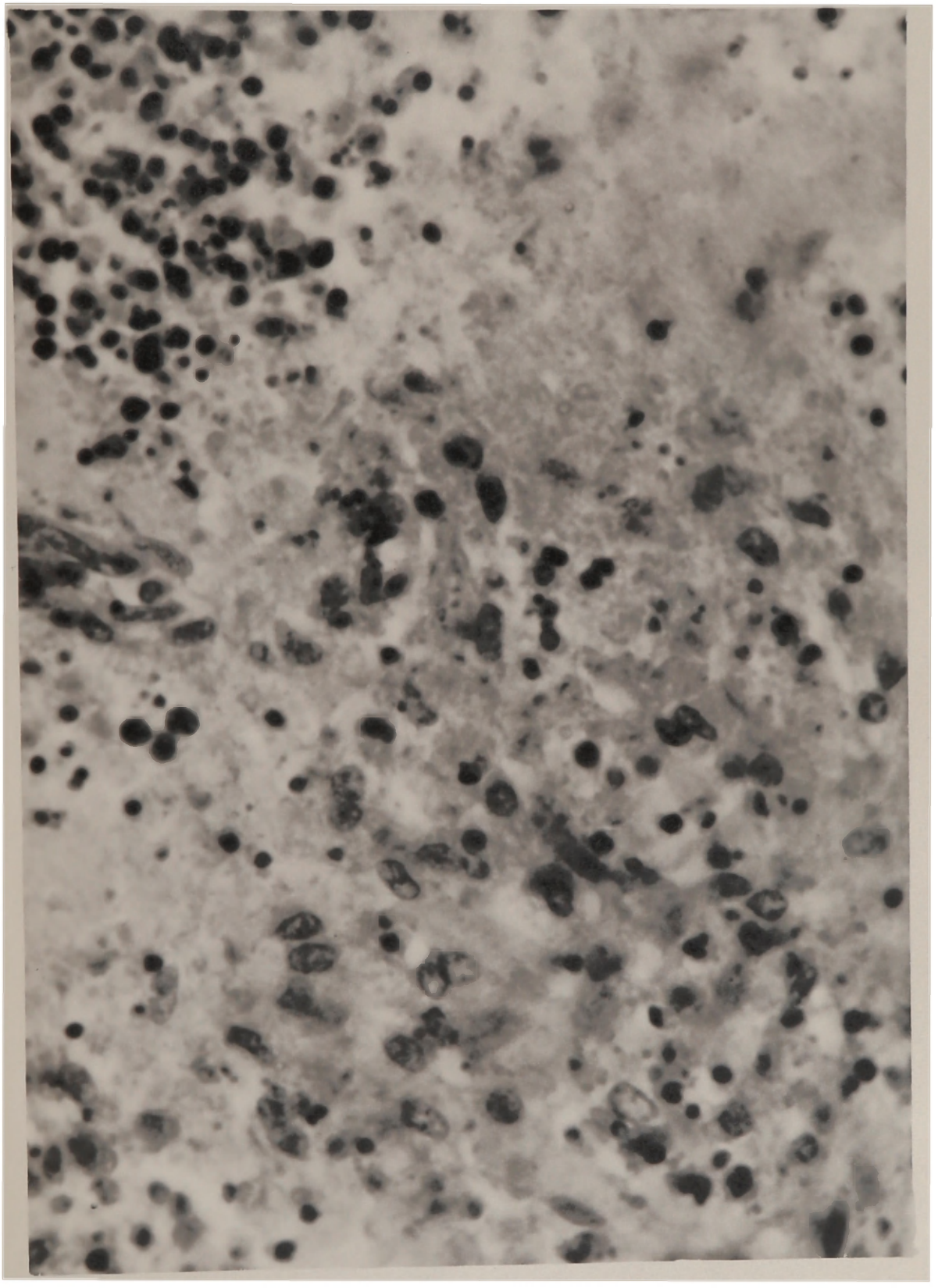
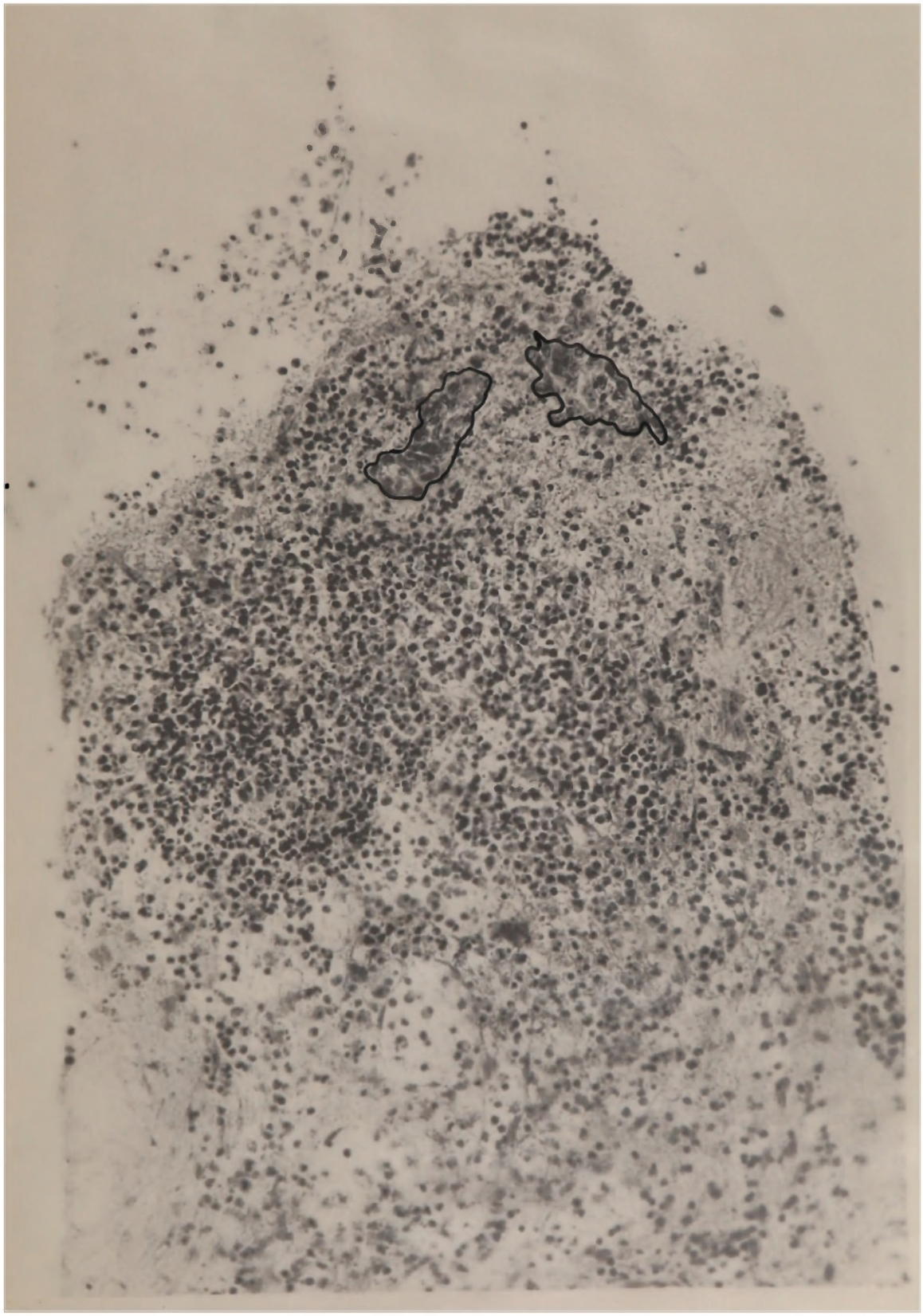
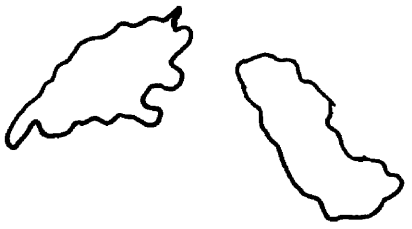


Plate XV. Section of a two-day-old culture. Reaction can be seen in the area marked. Mag, 400x

PLATE XV





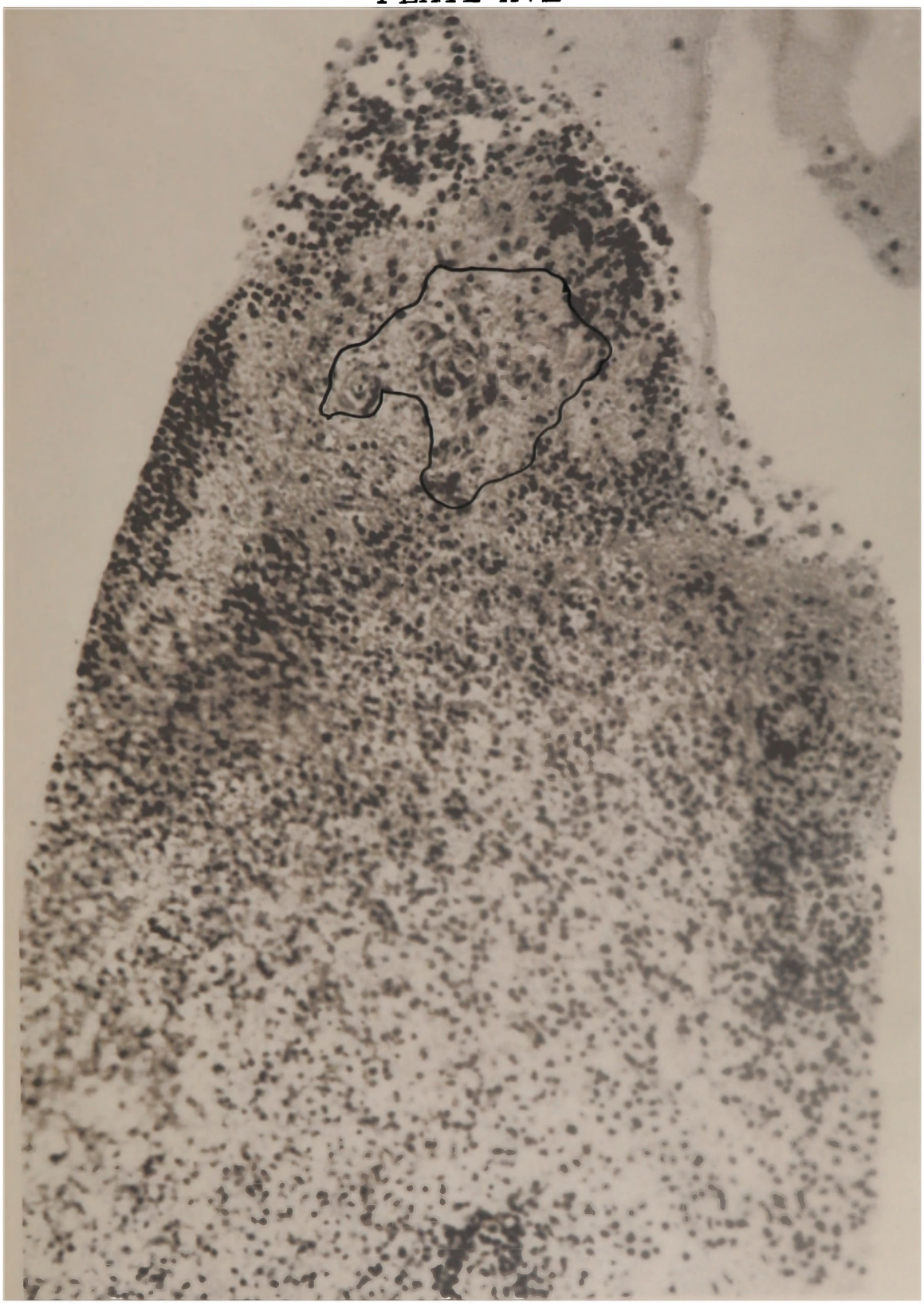
Section of a two-day-old culture. Reaction can be seen in the area marked Mag 400x

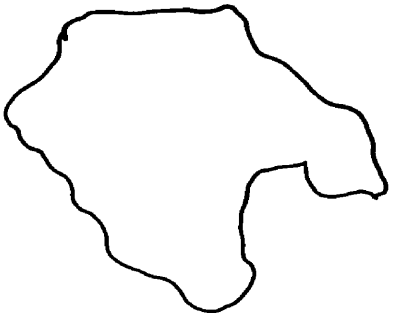
PLATE XV

Plate XVI. Section of an uninoculated control culture two days old. Mag. 400x

Plate XVII. Section of three-day-old culture. Reaction can be seen in the area marked. Mag. 400x

PLATE XVII

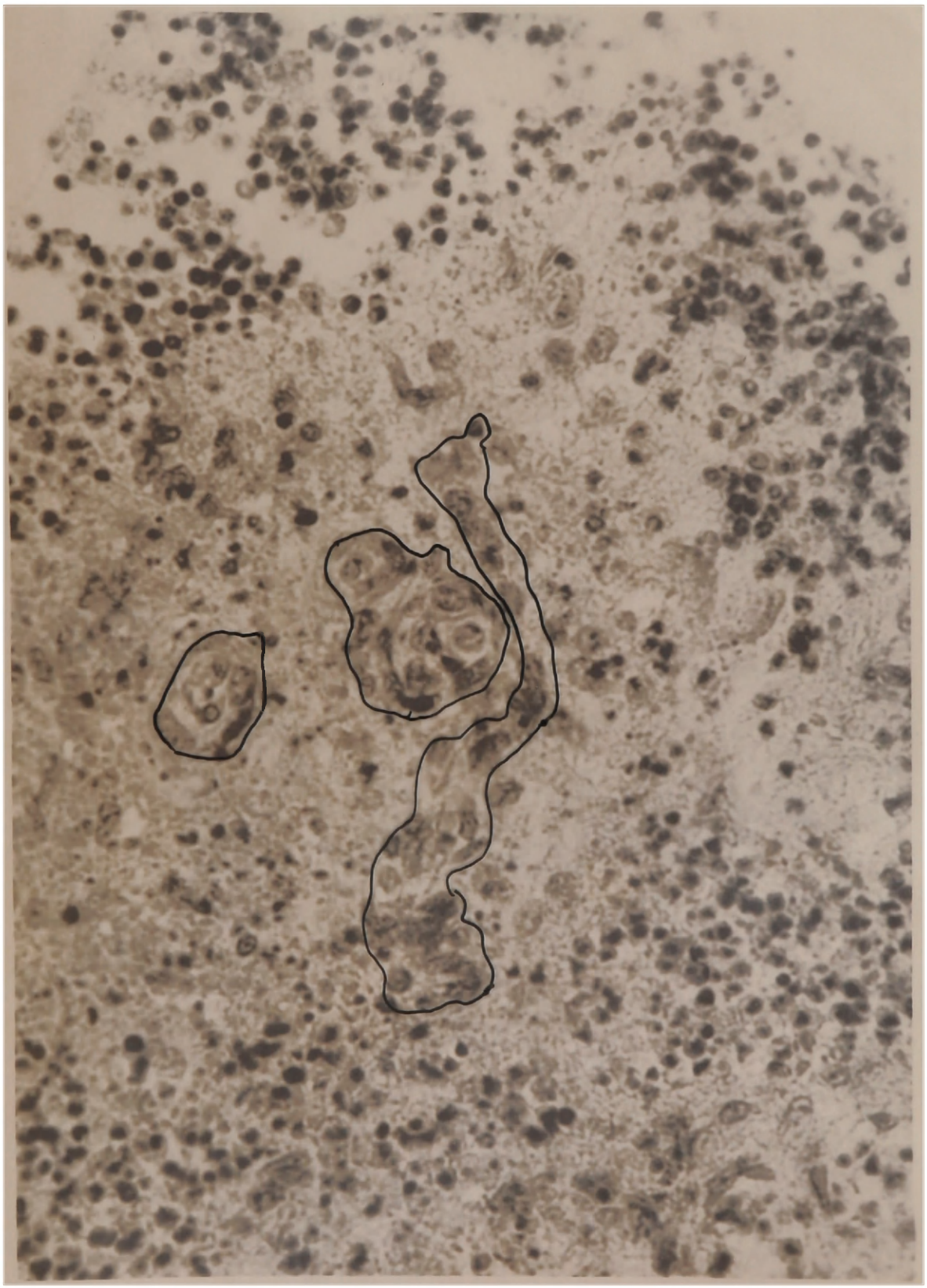




Section of three-day-old culture. Reaction can be seen in the area marked. Mag. 400x

Plate XVIII. High power of section in Plate XVII. Note the proliferation and infiltration of the epithelioid cells into the necrotic tissue. Mag. 1200x

PLATE XVIII



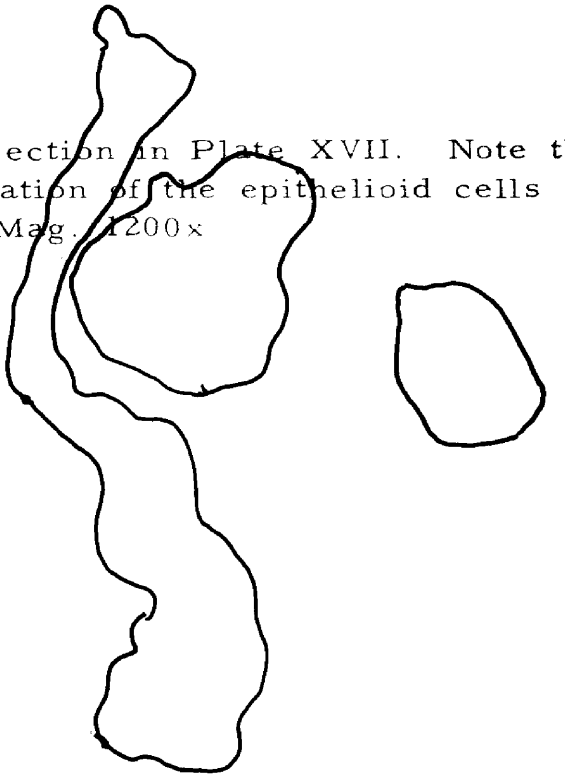


Plate XVIII. High power of section in Plate XVII. Note the proliferation and infiltration of the epithelioid cells into the necrotic tissue. Mag. 1200×

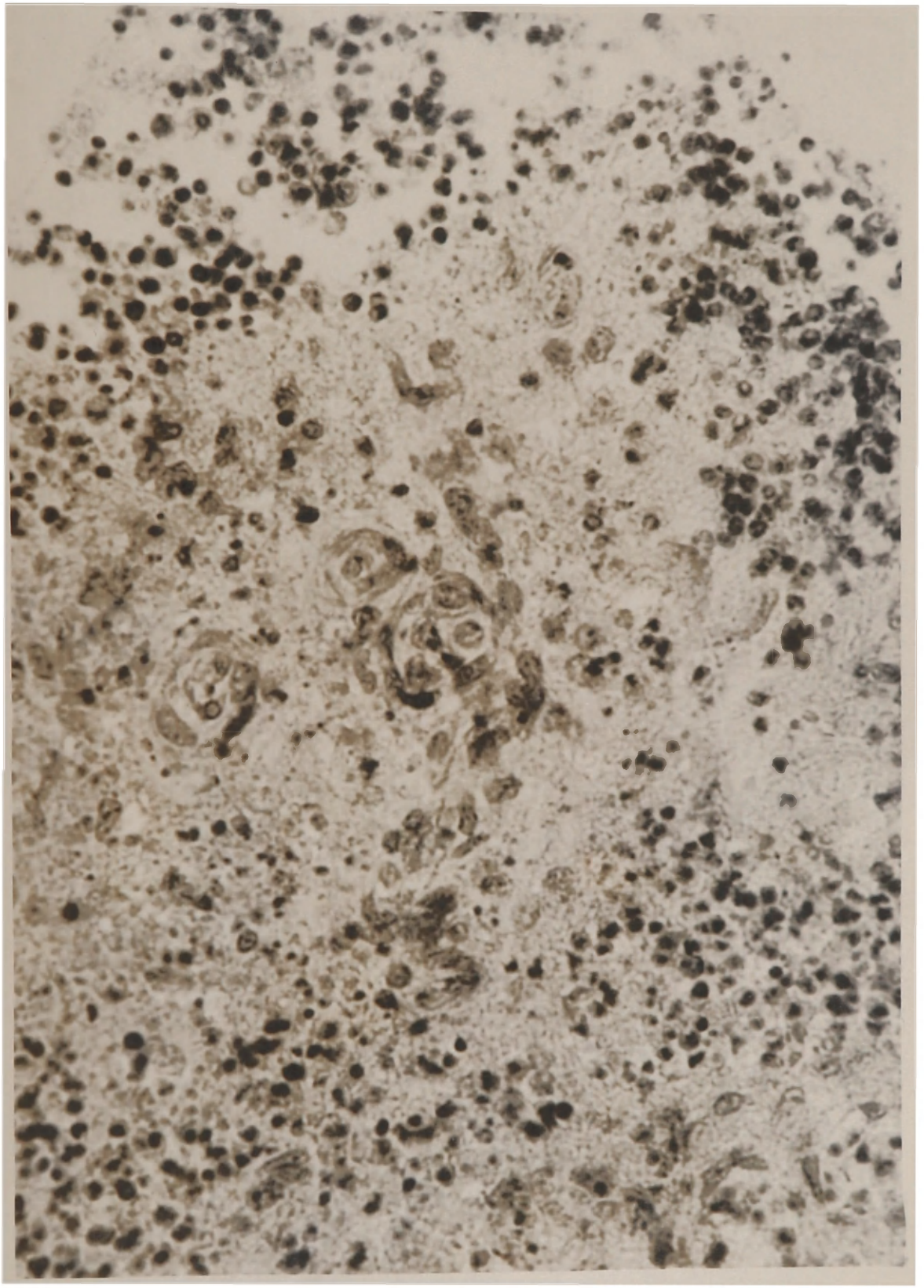
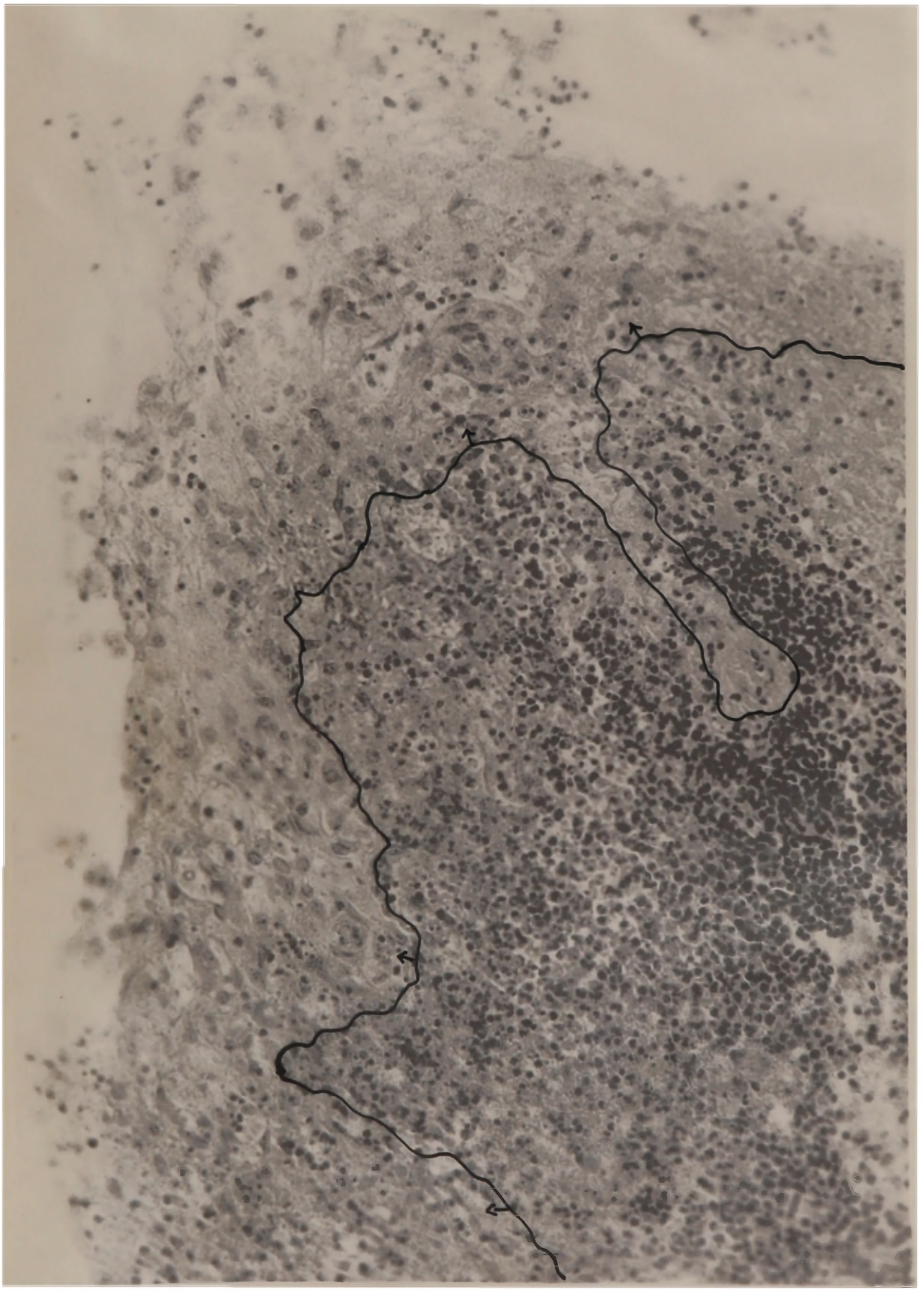
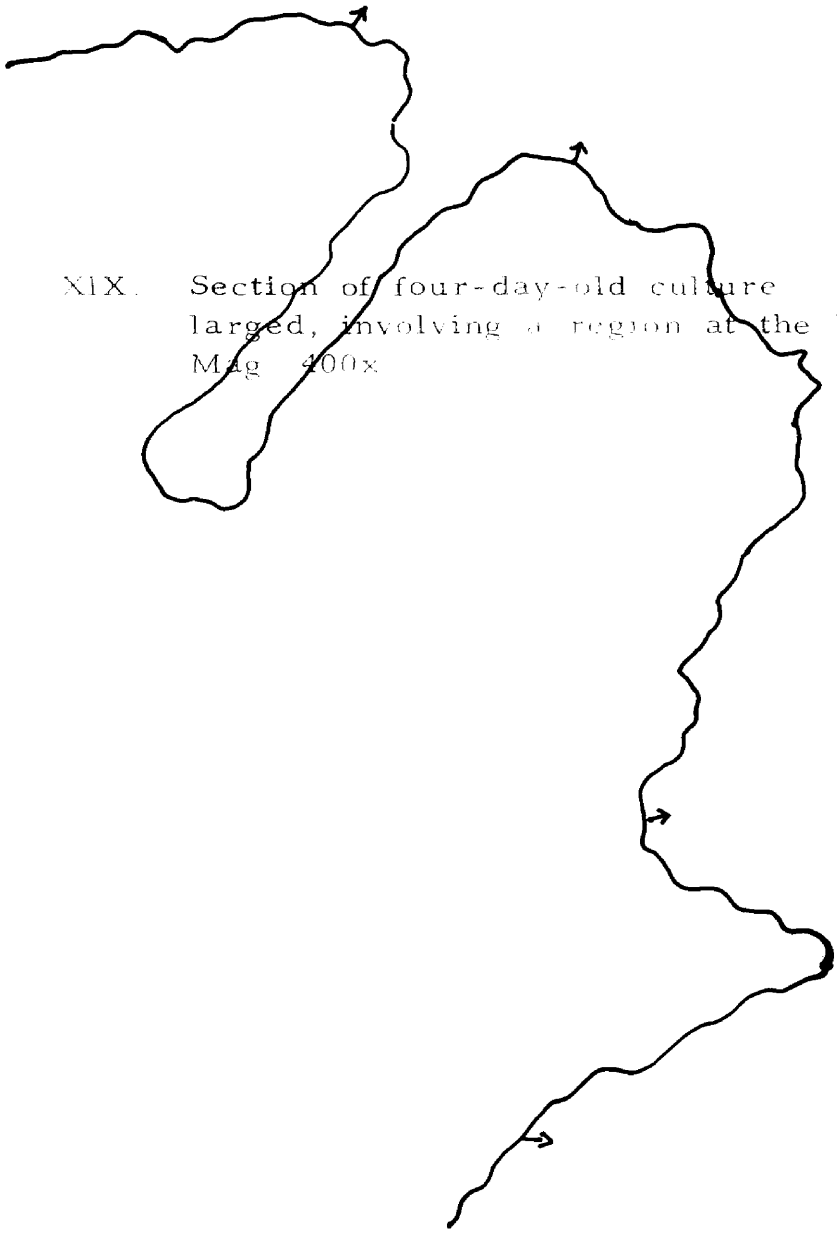


Plate XIX. Section of four-day-old culture. Reaction area has enlarged, involving a region at the left side of the section. Mag. 400x

PLATE XIX





XIX. Section of four-day-old culture. Reaction area has enlarged, involving a region at the left side of the sect Mag. 400x.

Plate XX. High power of section in Plate XIX. Note infiltration. Mag. 1200x

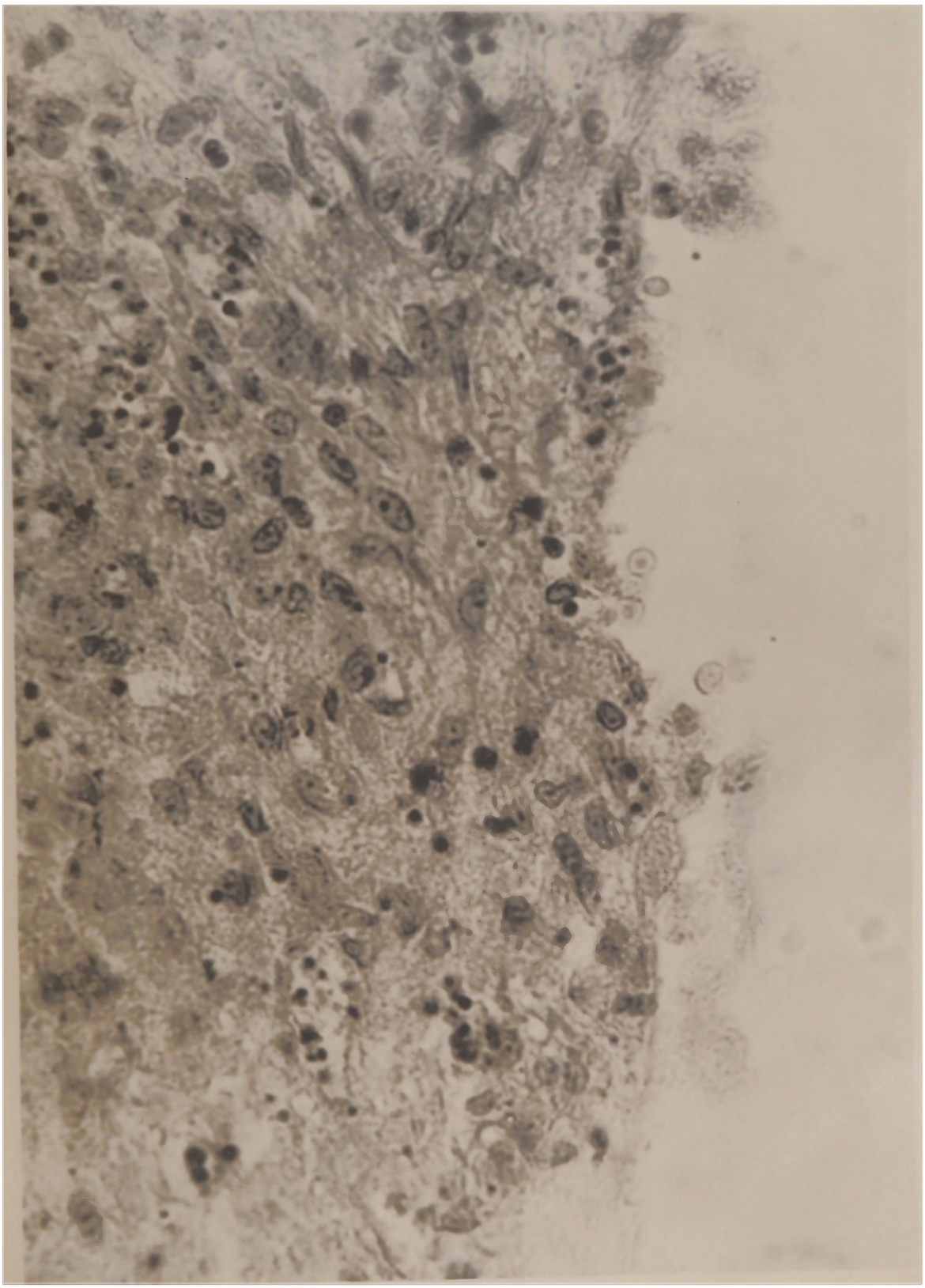


Plate XX. High power of section in Plate XIX. Note infiltration. Mag. 1200x

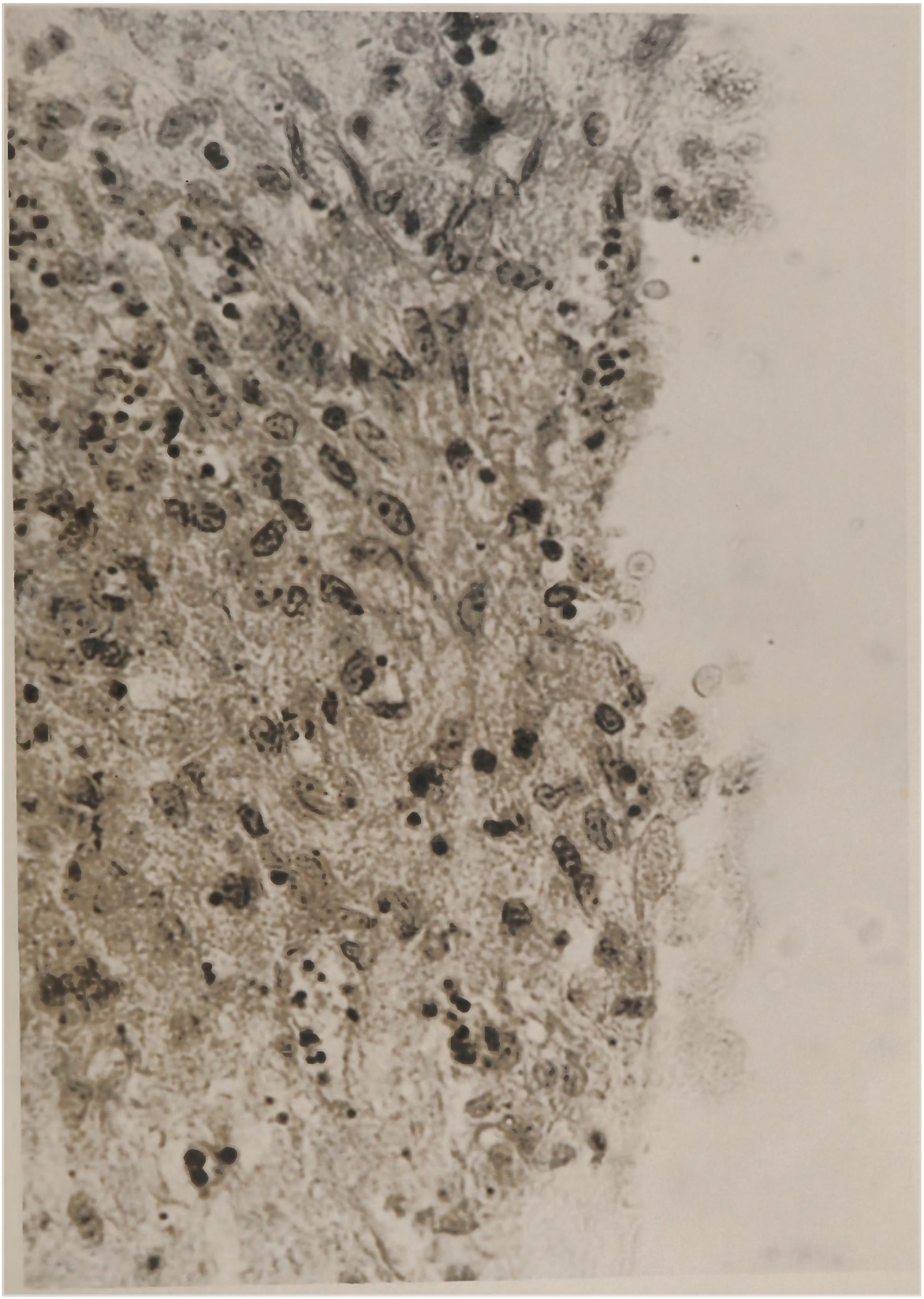


Plate XXI. Section of uninoculated control culture four days old. Mag. 400x

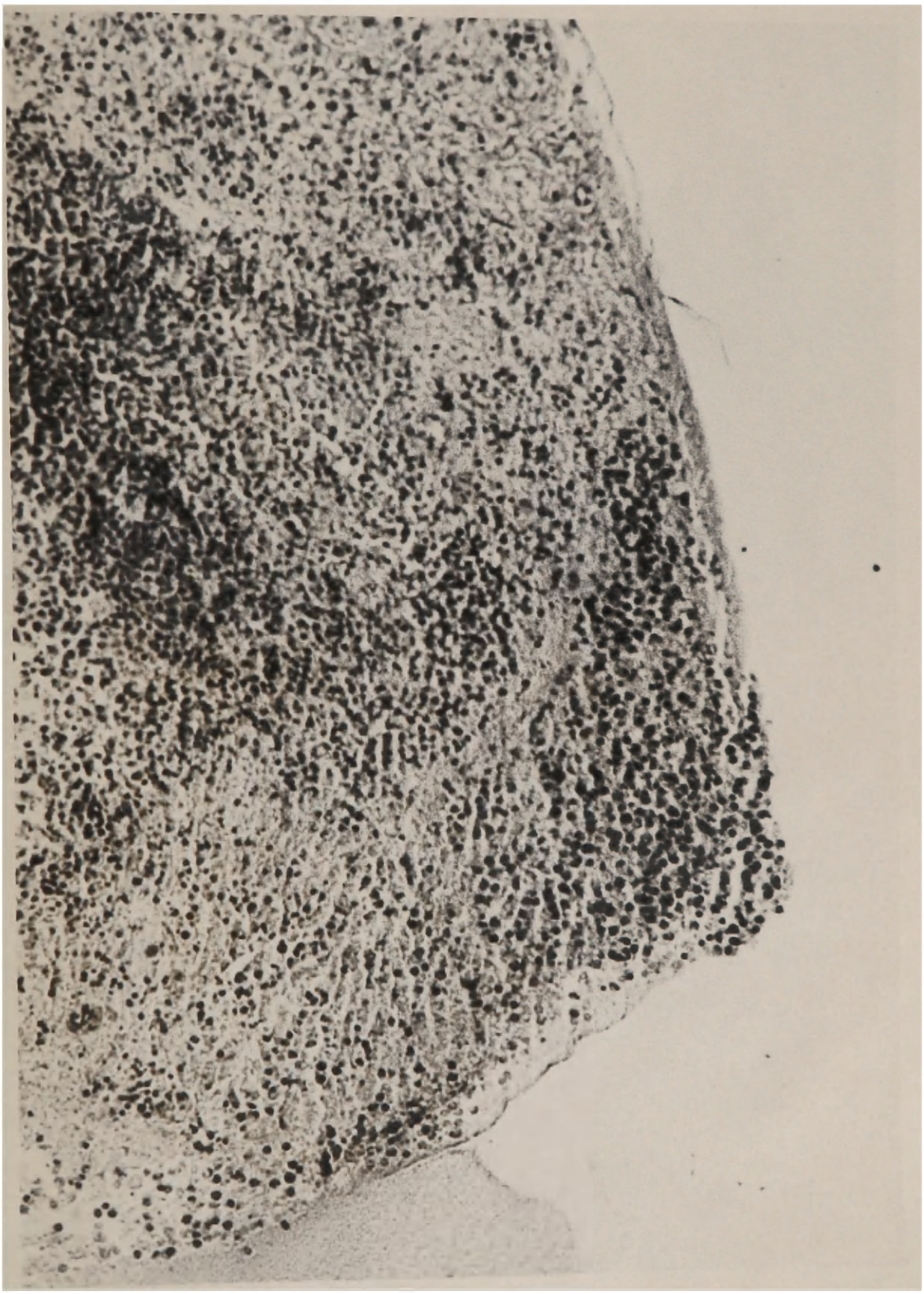


Plate XXII. Section of six-day-old culture. Area is of considerable size. Note marked infiltration. Mag. 400x

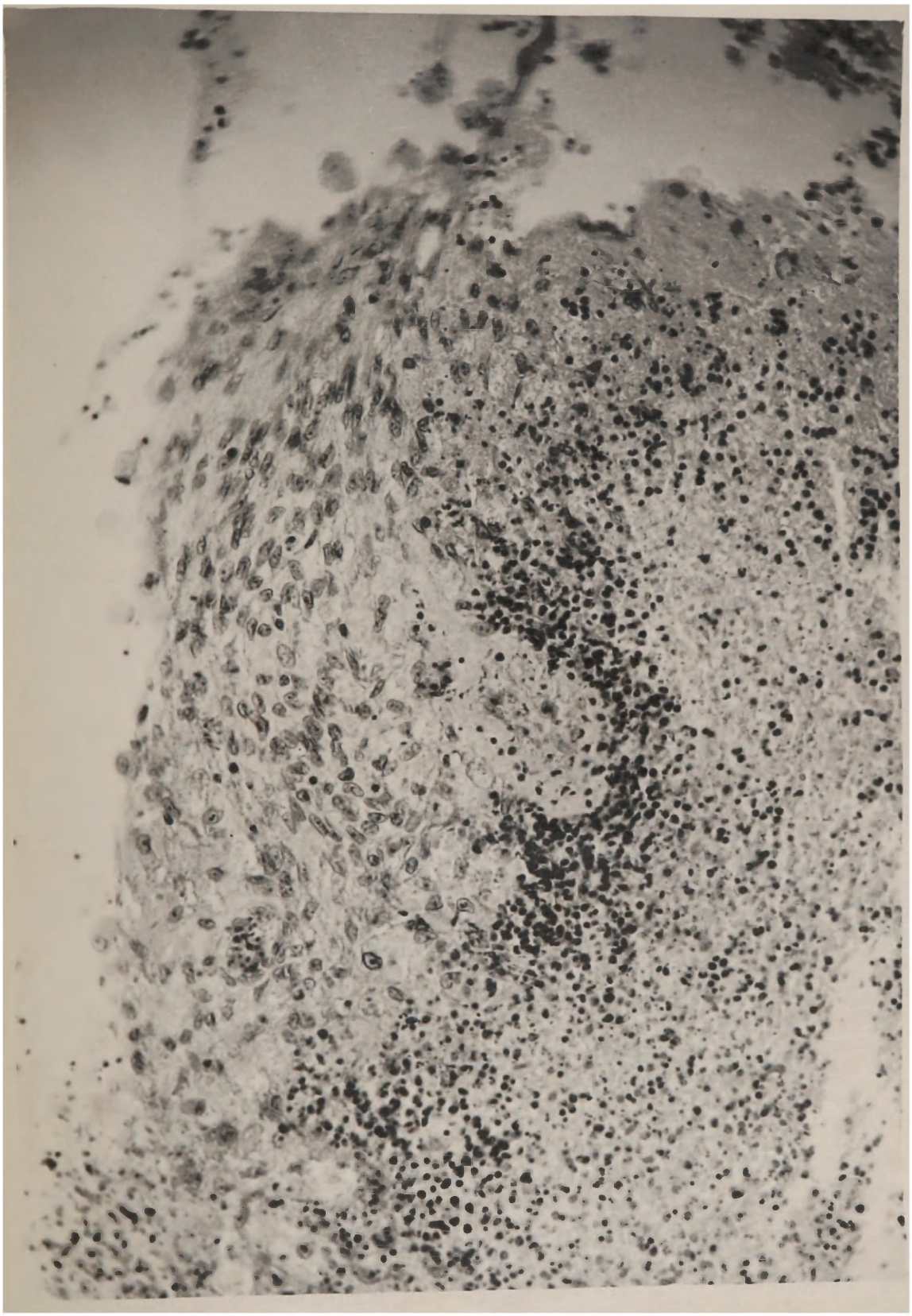


Plate XXIII. High power of six-day-old culture. Two circumscribed blood vessels are shown with proliferated cells. Note the large mononuclear cells on the periphery of the tissue. Mag. 1200x

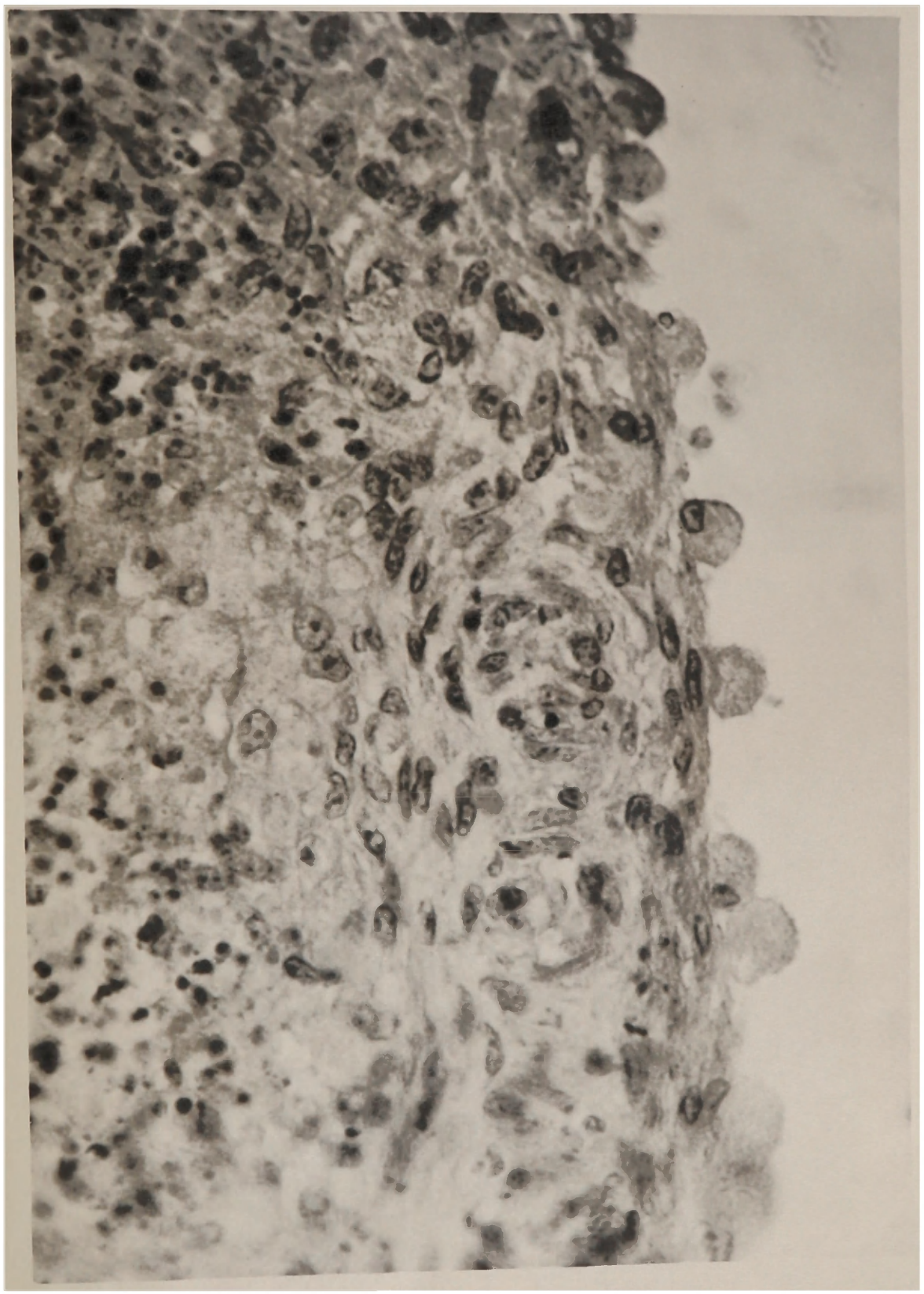


Plate XXIV. Infected cultures.

Figure 1. Same section as shown in Plate XXIII. Note the slight yellowish tinge in the cells of the upper left-hand corner. Mag. 1050x

Figure 2. Section stained with Weigert-Van Gieson. A green elastic tissue fiber can be seen around the lumen of the vessel. Mag. 800x

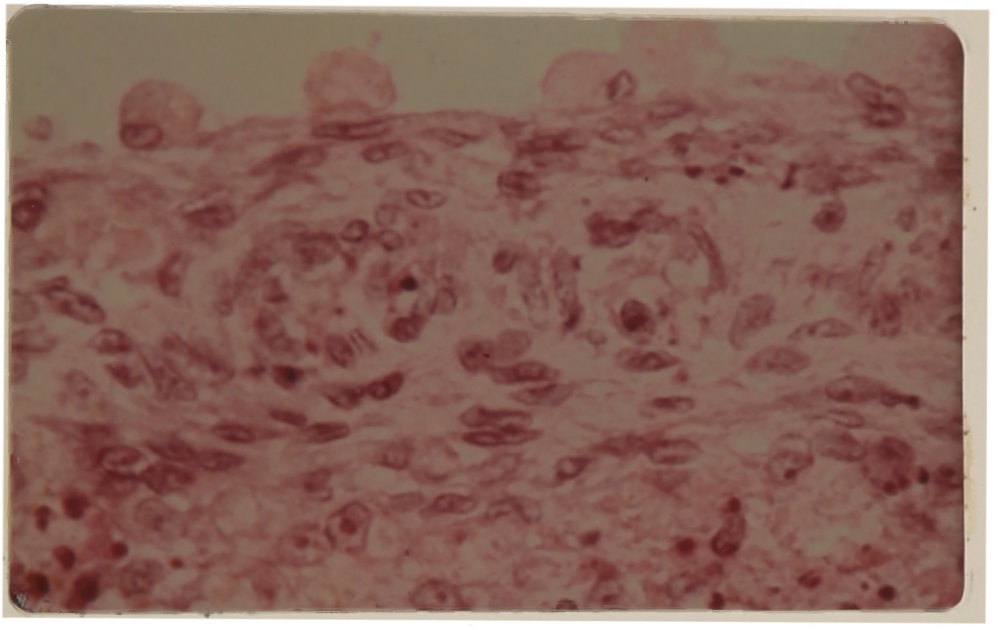


Figure 1

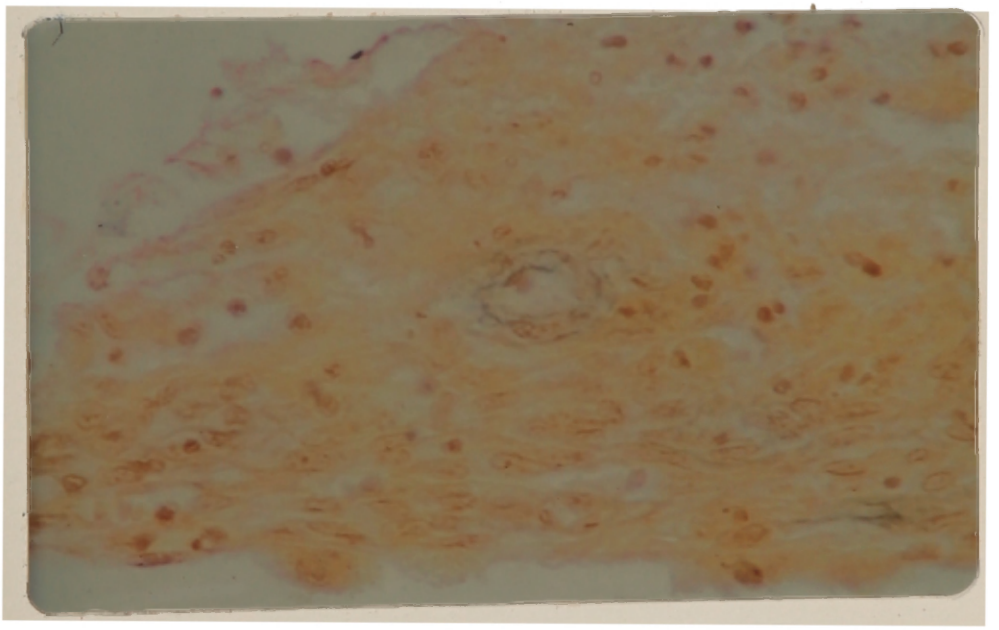


Figure 2

Plate XXV. Section of uninoculated control culture six days old. Mag. 400x

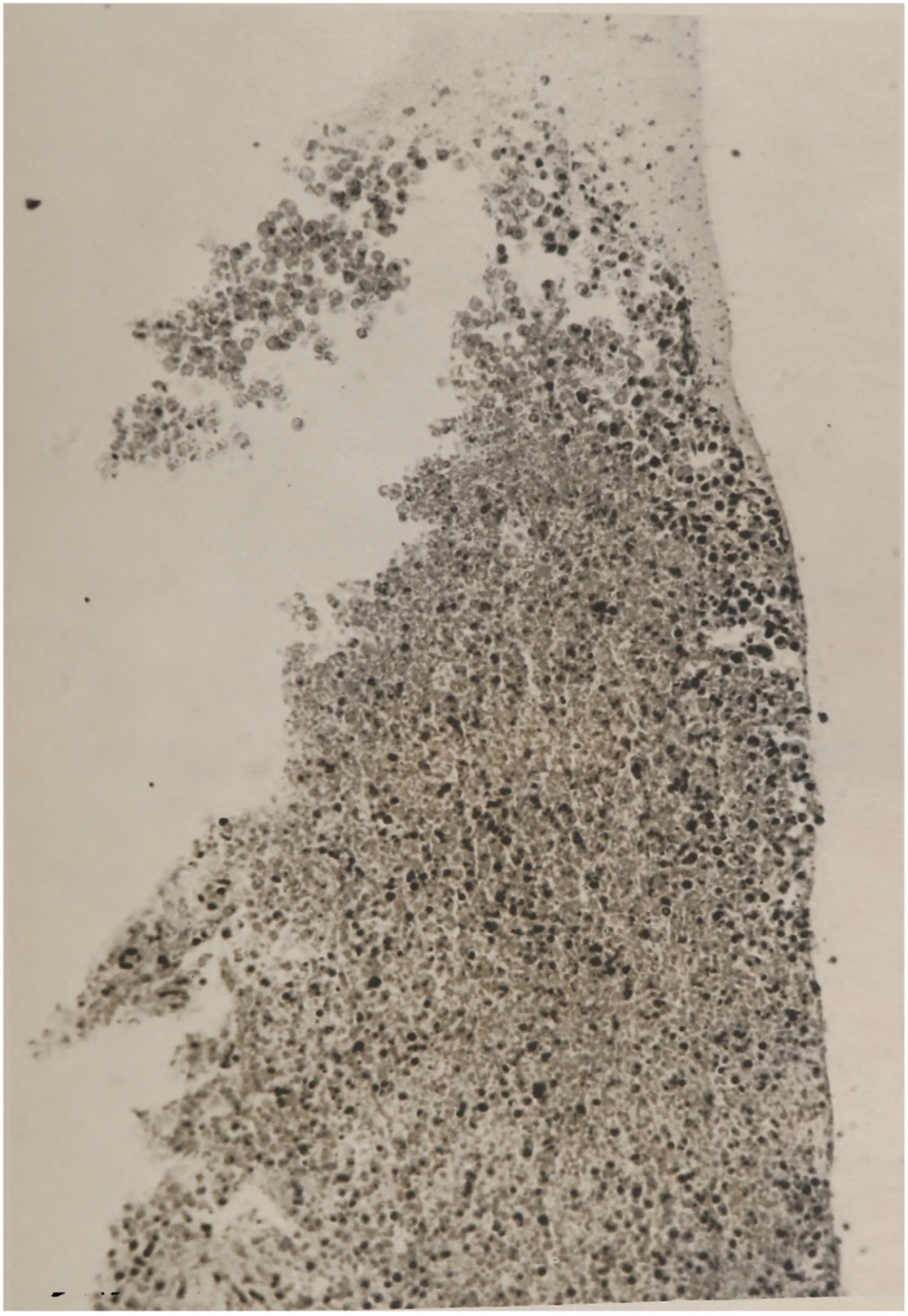
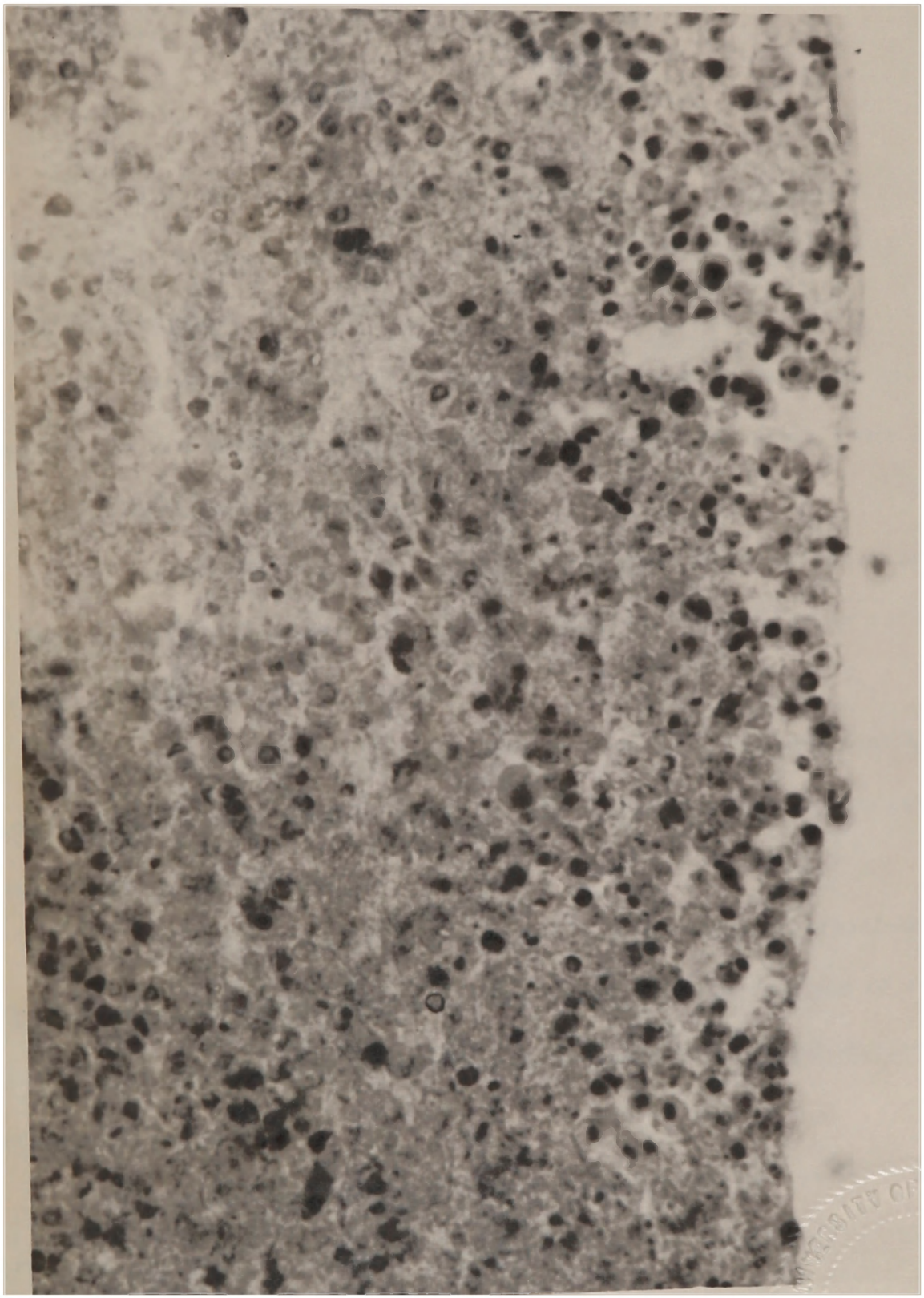


Plate XXVI. High power of section in Plate XXV. Mag. 1200x



SUMMARY AND CONCLUSIONS

1. The double coverslip of Maximow was used in a tissue culture study of the reaction of splenic explants inoculated with L. monocytogenes.

2. Medium 199, homologous plasma, and chicken embryo extract did not depress the growth of this organism after remaining in these fluids for seven days.

3. After 48 hours in tissue culture the bacteria could not be demonstrated in artificial media unless the tissues were refrigerated at 4°C for several days.

4. Observations of living tissue cultures revealed that L. monocytogenes was always motile. The organism did not suppress the growth of the tissue.

5. Stained whole mounts of the tissue cultures revealed cells which contained many bizarre nuclear forms characteristic of the granulomas produced in a disease of the newborn, granulomatosis infantiseptica.

6. Serial sections of infected tissue cultures revealed a granulomatous inflammatory reaction. The cells responsible for this reaction appeared to arise from vascular sinus endothelium. This

reaction was similar to that described for granulomatosis infantiseptica.

7. It is not known at this time if the granulomatous response seen in the cultured tissue was specific for L. monocytogenes or if it was produced as a result of a nonspecific irritant.

LITERATURE CITED

Allgöwer, M., and H. Block. 1949. The effect of tubercle bacilli on the migrations of phagocytes in vitro. Am. Rev. Tuberc. 59: 562-566.

Aronson, J. D. 1931. The specific cytotoxic action of tuberculin in tissue culture. J. Exp. Med. 54: 387-397.

Baker, L. E., and A. Carrel. 1926. Action on fibroblasts of the protein fraction of embryonic tissue extract. J. Exp. Med. 44: 387-395.

Baker, L. E., and A. A. Ebling. 1939. Artificial maintenance media for cell and organ cultivation. I. The cultivation of fibroblasts in artificial and serumless media. J. Exp. Med. 69: 365-378.

Bloom, William. 1928. Origin and nature of the monocyte. Folia Hematologia. 37: 1-62.

Bloom, William. 1928a. The formation of abscesses in an infection with Bacterium monocytogenes. Arch. Path. 6: 995 1007.

Burn, C. G. 1934. Unidentified gram positive bacillus associated with meningo-encephalitis. Proc. Soc. Exp. Biol. & Med. 31: 1095-1097.

Burn, C. G. 1935. Characteristics of a new species of the genus Listerella obtained from human sources. J. Bact. 30: 573-591.

Burn, C. G. 1936. Clinical and pathological features of an infection caused by a new pathogen of the genus Listerella. Am. J. Path. 12: 341-348.

Burrows, M. T. 1910. The cultivation of the tissues of the chick embryo outside the body. J. Am. Med. Assoc. 55: 2057-2058.

Carrel, A. 1912. The permanent life of tissues outside of the organism. J. Exp. Med. 15: 516-528.

Carrel, A. 1913. Artificial activation of the growth in vitro of connective tissue. J. Exp. Med. 17: 14-19.

Carrel, A. 1913a. Neue Untersuchungen über das Selbstandige Leben der Gewebe und Organe. Berlin. Klin. Wchnschr. 50: 1097-1011.

Carrel, A., and M. T. Burrows. 1910. Cultivation of adult tissues and organs outside the body. J. Am. Med. Assoc. 55: 1379-1381.

Coman, D. R. 1940. Chemotaxis of monocytes contrasted with that of polymorphonuclear leukocytes and lymphocytes. Arch. Path. 30: 896-901.

Conway, E. A. 1938. Reaction of lymphatic tissue in early stages of Bacterium monocytogenes infection. Arch. Path. 25: 200-227.

Conway, E. A. 1939. Reaction of lymphatic tissues of rabbits to repeated injections of Bacterium monocytogenes. J. Inf. Dis. 64: 217-240.

Cunningham, R. S., F. R. Sabin, and C. A. Doan. 1923. The differentiation of two distinct types of phagocytic cells in the spleen of the rabbit. Proc. Soc. Exp. Biol. & Med. 21: 330-333.

Dulbecco, R. 1952. Production of plaques in monolayer tissue cultures of single particles of an animal virus. Proc. Nat. Acad. Science. 38: 747-751.

Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with Poliomyelitis viruses. J. Exp. Med. 99: 167-199.

Erdmann, G., and J. Potel. 1953. Listeriose der Neugeborenen: Granulomatosis infantiseptica. Zeitschrift f. Kinderheilkunde 73: 113-132.

Evans, V. J., and W. L. Earle. 1947. The use of perforated cellophane for the growth of cells in tissue culture. J. Nat. Cancer Inst. 8: 103-119.

Evans, V. J., W. R. Earle, K. K. Sanford, J. E. Shannon, and H. K. Waltz. 1951. The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907-922.

Fischer, A. 1941. The nature of the growth-promoting substances in the embryonic tissue juice. Acta. Physiol. Scand. 3: 54-70.

Fischer, A. 1946. Biology of tissue cells, Copenhagen, Gyldendal. in Methods of Tissue Culture. R. C. Parker, 2nd Ed., Paul B. Hoeber, New York, N. Y., p. 121.

Fischer, A., T. Astrup, G. Ehrensuard, and V. Oehlenschlager. 1948. Growth of animal tissue cells in artificial media. Proc. Soc. Exp. Biol. & Med. 67: 40-46.

Földvari, Francis. 1932. The conduct of Spirocheta pallida in tissue explantations. Am. J. Syph. Gonor. Ven. Dis. 16: 145-154.

Foot, Nathan C. 1925. The endothelial phagocyte. A critical review. Anat. Rec. 30: 15-51.

Forbus, Wiley D. 1943. Reaction to Injury. Pathology for Students of Disease. Williams and Wilkins Co., Baltimore.

Forbus, Wiley D. 1949. Granulomatous Inflammation: Its Nature, General Pathological Significance and Clinical Character. Monograph in American Lectures in Pathology. 53 pages. Charles C. Thomas, Springfield, Illinois.

Forkner, C. E. 1930. The origin of the monocytes in certain lymph nodes, their genetic relation to other connective tissue cells. J. Exp. Med. 52: 385-401.

Gey, G. O. 1933. An improved technic for massive tissue culture. Am. J. Cancer 17: 752-756.

Girard, Kenneth F., and E. G. D. Murray. 1951. Listeria monocytogenes as the cause of disease in man and animals and its relations to infectious mononucleosis from an etiological and immunological aspect. Am. J. Med. Sciences 221: 243-352.

Gray, M. L. 1954. Listeria monocytogenes: A review and colonial dissociation. Unpublished Ph.D. thesis. Michigan State College. 120 numb. leaves.

Gray, M. L., Chintamani Singh, and F. Thorp, Jr. 1955. Abortion, stillbirth and death of young of rabbits induced by Listeria monocytogenes. I. Ocular instillation. In Press.

Gray, M. L., Chintamani Singh, and F. Thorp, Jr. 1955a. Abortion, stillbirth and death of young of rabbits induced by Listeria monocytogenes. II. Oral exposure. In Press.

Gray, M. L., H. J. Stafseth, F. Thorp, Jr., L. B. Sholl, and W. F. Riley. 1948. A new technique for isolating Listerellae from the bovine brain. J. Bact. 55: 471-476.

Hagemann, U., and H. Simon. 1953. Fruchttod oder Fruhgeburt durch diaplacentare Listerieninfektion. Geburtshilfe und Frauenheilkunde 12: 1089-1095.

Hagemann, U., H. Simon, and A. Bienengräber. 1953. Die Listeriose der Fruhgeburten. Zentralblatt fur Allegemeine Pathologie 90: 17-23.

Hahnefeld, Horst. 1954. Ergebnisse von Fütterungsuersuchen an Tieren mit Listeria monocytogenes (Pinie) Prophylaxe: Zeitschrift fur Mikrobiologie, Epidemiologie, Hygiene und Sozialhygiene 4: 164-169.

Hahnefeld, H., and E. Nisolk. 1954. Über das Vorkommen der Listeriose in Ostmecklenburg Beobachtungen bei Neugeborenen. Deut. Gesundheitsw. 5: 149-155.

Harrison, R. G. 1907. Observation on the living developing nerve fiber. Proc. Soc. Exp. Biol. & Med. 4: 140-143.

Herzog, A. J. 1938. The phagocytic activity of human leukocytes with special reference to their type and maturity. Am. J. Path. 14: 595-604.

Jensen, Rue, and D. R. Mackey. 1949. Listerellosis in cattle and sheep. J. Am. Vet. Med. Assoc. 114: 420-424.

Julianelle, L. A., and Elizabeth Moore. 1942. Pathological changes in Listerella infection particularly of the eye. Am. J. Path. 18: 813-825.

Kast, C. C., and J. A. Kolmer. 1933. On the cultivation of Spirocheta pallida in living tissue media. Am. J. Syph. Gonor. Ven. Dis. 17: 529-532.

King, J. T., B. S. Green, and A. F. Henschel. 1928. Response of leukocytes to colonies of Streptococci growing in tissue culture media. Proc. Soc. Exp. Biol. & Med. 38: 812-815.

Lang, F. J. 1925. The reaction of lung tissue to tuberculous infection in vitro. J. Inf. Dis. 37: 430-442.

Lang, F. J. 1926. Role of endothelium in the production of polyblasts (mononuclear wandering cells) in inflammation. Arch. Path. 1:41-63.

Lang, F. J. 1928. Zur monozyten-frage. Folia Haematologia 36: 383-389.

Lewis, M. R. 1916. Sea water as a medium for tissue cultures. Anat. Rec. 10: 287-299.

Lewis, M. R. 1920. The formation of vacuoles due to Bacillus typhosus in cells of tissue cultures of the intestine of the chick embryo. J. Exp. Med. 31: 293-311.

Lewis, M. R., and W. H. Lewis. 1911. The cultivation of tissues from chick embryos in solutions of NaCl, $CaCl_2$, KCl and $NaHCO_3$. Anat. Rec. 5: 277-294.

Lewis, W. H. 1925. The transformation of mononuclear blood cells into macrophages, epithelioid cells and giant cells. Harvey Lectures 21: 77-112.

Ljunggren, C. A. 1897. Von der Fahigkeit des Hautepithels, ausserhalb des Organismus sein leben zubehalten, mit Berucksichtigung der Transplantation. Deutsch. Ztschr. f. Chir., 47: 608-615.

Loeb, L. 1902. On the growth of epithelium in agar and blood serum in the living body. J. Med. Res. 8: 109-115.

Mallory, F. B. 1898. A histological study of typhoid fever. J. Exp. Med. 3: 611-616.

Mallory, F. B. 1942. Pathological Technique. E. B. Saunders Co., Philadelphia.

Maximow, A. A. 1924. Tuberculosis of mammalian tissue in vitro. J. Inf. Dis. 34: 549-584.

Maximow, A. A. 1924a. Relation of blood cells to connective tissues and endothelium. Phys. Rev. 4: 533-563.

Maximow, A. A. 1925. Tissue cultures of young mammalian embryos. Carnegie Inst. Wash., Contrib. Embryol. 16: 47-113.

Maximow, A. A. 1927. Development of non-granular leucocytes (lymphocytes and monocytes) into polyblasts (macrophages) and fibroblasts in vitro. Proc. Soc. Exp. Biol. & Med. 24: 570-572.

McCutcheon, M., and H. M. Dixon. 1936. Chemotropic reactions of polymorphonuclear leucocytes to various micro-organisms. Arch. Path. 21: 749-755.

McJunkin, F. A. 1926. Rosette and granule-hyaline phagocytes in tissue cultures stained supravitally with Neutral Red. Proc. Soc. Exp. Biol. & Med. 24: 84-86.

Morgan, J. F., H. J. Morton, and R. C. Parker. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exp. Biol. & Med. 73: 1-8.

Murray, E. G. D. 1955. A characterization of listeriosis in man and other animals. The Canadian Med. Assoc. Jour. 72: 99-103.

Murray, E. G. D., R. A. Webb, and M. B. R. Swann. 1926. A disease of rabbits characterized by a large mononuclear leucocytosis caused by hitherto undescribed bacillus, Bacterium monocytogenes. J. Path. and Bact. 29: 407-439.

Nyfeldt, A. 1929. Etiologie de la mononucleose infectieuse. Compt. rend. Soc. de. Biol. 101: 590-592.

Nyfeldt, A. 1932. Klinische und Experimentelle Untersuchungen über die Mononucleosis infectiosa. Folia Hematologia 47: 1-144.

Osebold, John W., and Taka Inouye. 1955. Pathogenesis of Listeria monocytogenes infections in natural hosts. I. Rabbit studies. J. Inf. Dis. 95: 52-66.

Osebold, John W., and Taka Inouye. 1955a. Pathogenesis of Listeria monocytogenes infection in natural hosts. II. Sheep studies. J. Inf. Dis. 95: 67-78.

Perry, W. L. M. 1948. The cultivation of Treponema pallidum in tissue culture. J. Path. Bact. 60: 339-342.

Pons, C. A., and L. A. Julianelle. 1939. Isolation of Listerella monocytogenes from infectious mononucleosis. Proc. Soc. Exp. Biol. & Med. 40: 360-362.

Porter, K. R., A. Claude, and E. F. Fullam. 1945. A study of tissue culture cells by electron microscopy. Methods and preliminary observations. J. Exp. Med. 81: 233-246.

Potel, J. 1952. Zur Granulomatosis infantiseptica. Zentr. Bakt. Abt. I. Orig. 158: 329-332.

Potel, J. 1953. Ätiologie der Granulomatosis Infantiseptica. Wissen. Zeit. Martin Luther Univer. 2: 341-364.

Potel, J. 1954. Zur epidemiologie der Listeriose der Neugeborenen (Granulomatosis infantiseptica). Deuts. Gesundheitsw. 3: 92-95.

Rezzesi, F. D. 1933. La infezione da "Bacterium monocytogenes" e il problema del monocito. Folia Hematologia 14: 239-314.

Riess, H. J. 1953. Zur Pathologischen Anatomie der Kindlichen Listeriosis. Kinderarztliche Praxis. Wissenschaftlicher Verhandlungsbericht der Kinderarztetagung der Deutchen Demokratischen Republik in Leipzig 24. biz 26 April. pp. 92-100.

Robbins, F. C., T. H. Weller, and J. F. Enders. 1952. II. The propagation of the Poliomyelitis viruses in roller-tube cultures of various human tissues. J. Immunol. 69: 673-694.

Roux, W. 1923. Meine Entwicklungsmechanische methodik. In: Abderholden, E., Handbuch der biologischen Arbeitsmethoden. Berlin, Urban and Schwarzenberg, Abt. 5 Teil 3 A, 539-616.

Sabin, F. R., C. A. Doan, and R. S. Cunningham. 1925. Discrimination of two types of phagocytic cells in the connective tissues by the supravital technique. Contr. to Embry. Carnegie Inst. 16: 125-162.

Sanford, K. K., W. R. Earle, and G. D. Likely. 1948. The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229-246.

Schmidt, V., and A. Nyfeldt. 1938. Ueber Mononucleosis infectiosa und Meningoencephalitis. Acta Oto-laryng. 26: 680-688.

Seeliger, H., and G. Linsenmeier. 1953. Die listeriose und ihre Erreger (Listeriose monocytogenes). Zeitschr. f. Hygiene, 136: 335-378.

Simms, H. S. 1936. The effects of physiological agents on adult tissues in vitro. Science 83: 418-419.

Simms, H. S., and M. Sanders. 1942. Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses. Arch. Path. 33: 619-635.

Simms, H. S., and N. P. Stillman. 1937. Substances affecting adult tissue in vitro. I. The stimulating action of trypsin on fresh adult tissue. J. Gen. Phys. 20: 603-619.

Simms, H. S., and N. P. Stillman. 1937a. Substances affecting adult tissue in vitro. II. A growth inhibitor in adult tissue. J. Gen. Phys. 20: 621-629.

Simon, H. 1953. Über die Listerienenzephalitis. Zent. Allg. Path. u. Path. Anat. 90: 353-359.

Smith, D. T., H. S. Willis, and M. R. Lewis. 1922. The behavior of cultures of chick embryo tissue containing avian tubercle bacilli. Am. Rev. Tuberc. 6: 21-38.

Smyth, H. F. 1915. The influence of bacteria upon the development of tissues in vitro. Zentr. Bakt. Abt. I. Orig. 76: 12-23.

Smyth, H. F. 1916. The reactions between bacteria and animal tissues under conditions of artificial cultivation. J. Exp. Med. 23: 265-283.

Tannenberg, J. 1929. I. Über die Entwicklungspotenzen der Fibroblasten in der Gewebekultur. II. Das Verhalten von Fibroblastenreinkulturen bei bakterieller Infektion. Arch. Exper. Zellforsh. 9: 528-568.

Vogelaar, J. P. M., and E. Erlichman. 1933. A feeding solution for cultures of human fibroblasts. Am. J. Cancer 18: 28-38.

Wallbach, Günter. 1934. Sur la monocytose infectieuse du lapin par le bacille monocytogene. Arch. d'Anat. Micro. 30: 275-294.

Webb, R. A. 1943. Listerella monocytogenes isolated from a case of infectious mononucleosis. Lancet 2: 5-10.

Youngner, J. S. 1954. Monolayer tissue cultures. I. Preparation and standardization of suspension of trypsin-dispersed monkey kidney cells. Proc. Soc. Exp. Biol. & Med. 85: 202-205.

Youngner, J. S. 1954a. Monolayer tissue cultures. II. Poliomyelitis virus assay in roller-tube cultures of trypsin-dispersed monkey kidney. Proc. Soc. Exp. Biol. & Med. 85: 527-530.