ATTEMPTS AT AXENIC CULTIVATION OF *HISTOMONAS MELEAGRIDIS*

by

DIANE S. HIRSCH

B.A., University of Hawaii, Honolulu, Hawaii, 1970

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Parasitology

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1975

Approved by:

M.F. Hansen
Major Professor

TABLE OF CONTENTS

RESULTS

INTRODUCTION

It has been suggested that Histomonas meleagridis is dependent upon one or more strains of cecal bacteria for survival (Delappe, 1953; Franker and Doll, 1964; Bradley, 1965; Bradley and Reid, 1966; Lesser, 1964b), however, the nature of this dependence remains unknown. The concept of dialysis systems seemed an ideal way for studying interactions of this protozoan and cecal bacteria.

The present study was directed toward developing a method for axenic cultivation of H. meleagridis in order to conduct future studies on the effects of metabolic products of cecal bacteria using a dialysis system. Such a system would enable determination of whether the protozoan was utilizing bacterial by-products or ingesting bacteria per se.

Although Lesser (1961) reported a method for obtaining bacteria-free in vitro cultures of Histomonas, I could not repeat his results. Various methods for purification of this protozoan are presented in this thesis. While some methods for purification were successful, all attempts to cultivate bacteria-free histomonads in vitro failed. However, possibilities of alternative methods for purification and possible successful cultivation are described in the Discussion.

LITERATURE REVIEW

General Description and Life Cycle of Histomonas meleagridis:

Histomonas meleagridis (Smith, 1895) Tyzzer, 1920, a parasite of gallinaceous birds, particularly turkeys and pheasants, is a unicellular organism belonging to Phylum Protozoa, Class Mastigophora, Order Rhizomastigida. It ranges in size from 5 to 30 microns, has one flagellum but can display ameboid movement (Soulsby, 1968). Tyzzer (1919) described a cyst stage which since has not been reported by other researchers. A highly virulent strain will kill 100 per cent of infected turkeys. Typical pathognomonic symptoms are yellowish diarrhetic feces, loss of appetite, droopy wings, and ruffled feathers.

In nature the parasite is transmitted by means of embryonated eggs of Heterakis gallinarum, the common cecal nematode of domestic turkeys and chickens (Graybill and Smith, 1920). The histomonads are only mildly pathogenic in chickens and they serve primarily as a reservoir host (Soulsby, 1968). Tyzzer and Collier (1925) reported that turkeys may become infected with the histomonad by ingesting recently discharged feces containing the parasite. This mode of transmission was again reported by Horton-Smith and Long (1956). Lund, et al. (1966) reported that earthworms are capable of transmitting heterakid eggs containing the histomonads. The infection can be artificially induced by feeding turkeys male heterakids per os (Springer, et al., 1969) or by infecting birds rectally with cultures of the protozoa or ground infective tissues (Farmer and Stephenson, 1949).

The parasite survives in eggs of heterakids (Tyzzer and Fabyan,

1921; Farr, 1956, 1961; Gibbs, 1958, 1962; Lee, 1969) and emerges when the heterakid larvae reach the ceca. The histomonads penetrate the cecal wall producing ulcerations and greatly enlarged ceca. Ultimately, a hard core is formed in the lumen of the cecum. Some histomonads are carried via the hepatic portal circulation to the liver where they destroy hepatic tissue killing the host.

Cultivation of Histomonas meleagridis:

In vitro cultivation of the protozoan with a mixture of bacteria was first reported by Drbohlav (1924). He cultivated histomonads on an albumin slant overlaid with blood bouillon; a medium developed by Boeck and Drbohlav (1925) for the cultivation of Endamoeba histolytica. In 1934 Tyzzer cultured histomonads from intestinal contents and feces on a buffered egg albumin agar slant covered with 5 per cent horse serum in saline containing small quantities of sterile rice starch and charcoal. Bishop (1938) cultured protozoa obtained from liver lesions using inspissated horse serum slants developed by Dobell and Laidlaw (1926). Viable protozoa were observed after 24 hours, but cultures also had a mixed bacterial growth. De Volt and Davis (1936) used a slightly modified Locke-egg-serum medium of Tyzzer (1934) in which horse serum was replaced by turkey serum. Later De Volt (1943) discovered that bacteria in cultures fragmented the slant resulting in a colloid that shortened the growth period of cultures. He observed that the protozoa did not obtain any particular sustenance directly from the slant, so the slant was eliminated from culture. This new totally liquid medium was completely autoclavable. Sterile rice starch as a nutritional source was added to this medium prior to inoculation of protozoa obtained from

an infected bird.

In 1953 Delappe incorporated antibiotics in his medium to ascertain if antibiotics were effective in aiding the establishment of Histomonas in vitro immediately after isolation from in vivo sources. Twenty-four out of twenty-six cultures with antibiotics showed viable histomonads. He also noted that the addition of antibiotics to cultures prolonged the viability of the protozoa. Too great a decrease in the bacterial flora, however, resulted in the histomonads' inability to survive. The antibiotics employed were penicillin and streptomycin (100 or 150 units/ml) used either singly or conjointly, but attempts to free histomonad cultures of the entire bacterial flora were unsuccessful.

Lesser (1960) described a new medium for cultivation of histomonads. It consisted of an available mixture 199 (Microbiological Associates) diluted 1 to 10 with distilled water and 1 ml 5% $NaHCO_3$/100 ml, then sterilized by Seitz filtration. To this medium was added cream, antibiotics, mycostatin and a mixed bacterial flora. The growth reported was two to three organisms per microscopic field. Bradley (1963) reported growth of Histomonas in avian embryos but also reported that the growth achieved was highly variable.

Recently, Dwyer (1970) reported an improved method for the cultivation of histomonads. His medium consisted of 75% M199 mixture (Microbiological Associates), 10% heat inactivated normal horse serum, 5% chick embryo extract and 10-12 mg sterile rice starch in 10 ml medium per culture tube. The formulation used in our laboratory is one provided by McDougald (pers. comm.) which is the same as that provided by Dwyer except that the M199 concentration is 85% (Grand Island Biological Company).

Another medium used in our laboratory is a modified De Volt's medium (1943). This medium was modified by using all glass double distilled, double deionized water. As suggested by De Volt, the serum concentration was reduced from 2% to 1% and the glucose was deleted from the original formulation in order to prevent extensive bacterial growth.

Relationship of Histomonas with Cecal Bacteria:

Some studies on the relationship between Histomonas and cecal bacteria have been done using gnotobiotic turkeys and antibiotic treated cultures of histomonads.

In 1963 Doll, et al., devised a method for surface sterilizing invertebrate eggs, among which were Heterakis gallinarum eggs. Eggs were treated with low concentrations of peracetic acid without impairing viability. When administered to turkeys harboring normal flora, typical symptoms of histomoniasis were manifested.

Franker and Doll (1964) infected turkeys harboring single species of bacteria with sterile heterakid eggs containing the histomonads. Those birds harboring Bacillus cereus or Lactobacillus fermenti showed no signs of histomoniasis. One bird out of eight with Streptococcus faecalis exhibited histomoniasis. Liver and cecal involvement was seen in fifty percent of the birds harboring Escherichia intermedia, but symptoms were less severe than infections in conventional turkeys.

Lesser (1964a) investigated cultivation of antibiotic treated histomonads in conjunction with three different mixed bacterial flora. When cultured with Mongolian gerbil fecal pellets, only one out of ten cultures containing viable histomonads could be transferred twenty-two consecutive times. Three out of twelve cultures could be transferred

twenty-two times with hamster fecal pellets. With chick cecal bacteria five out of seven cultures could be maintained twenty-two times.

Another study by Lesser (1964b) dealt with *in vitro* growth of *Histomonas* using single species of bacteria. His analysis of bacterial flora from cecal droppings indicated four species of bacteria: *Proteus mirabilis*, *Escherichia freudii*, *E. coli* and a *Lactobacillus* (T16). Histomonads were maintained with individual bacterial species and transferred three times weekly for twenty-two times. The best survival indicated was histomonads cultured with *Escherichia freudii*. *Lactobacillus* did not support the protozoa, while other species supported them only through a few transfers.

In 1965 Bradley, et al., reported that histomonads inoculated with *Escherichia coli* into gnotobiotic turkeys caused liver lesions. When histomonads were inoculated in combination with *Aerobacter cloacae* or *Streptococcus fecalis*, no characteristic lesions were produced. Histomonads and *Bacillus subtilis* caused cecal ulcerations but not liver involvement. Apparently, some relationship exists between *Histomonas* and various strains of bacteria, particularly of the genus *Escherichia*. In 1966 Bradley and Reid investigated additional bacteria. No disease was produced by the parasite in conjunction with *Proteus mirabilis*. No lesions were demonstrated when viable histomonads were administered with killed *E. coli*. If *B. subtilis* in combination with *Clostridium perfringens* was administered with histomonads, symptoms of infection much like those of histomonads and *E. coli* were demonstrated.

Relationship of Other Protozoa and Bacteria:

Previous studies have suggested that bacteria associated with

protozoan cultures are important in their role as possible endosymbiotes within protozoan cells. In 1962 Gill and Vogel demonstrated that the bipolar bodies reported by Pyne (1961) in Crithidia oncopelti were bacterial symbiotes. The lysine present within the protozoan was synthesized by a bacterial pathway and not by a protozoan pathway. They also showed that this synthesis was localized in the endosymbiote and that the cytoplasm of the protozoan lacked the necessary enzymes required for synthesizing lysine by either pathway.

Another study proposing the role of bacteria as being an endosymbiote was that of Bonhomme-Florentin (1971). Electron microscopy indicated intracellular bacteria in the ectoplasm and bacteria in vacuoles of Entodinium. Using various methods, digestion of cellulose by Entodinium was observed, possibly due to the presence of bacteria.

Phillips et al., (1955) reported results with Entamoeba histolytica similar to those reported by Franker and Doll (1964) and Bradley, et al. (1965) for Histomonas meleagridis. Phillips and his co-workers microisolated cysts of the amebae free of bacteria to perform various studies using gnotobiotic guinea pigs. Bacteria-free hosts did not develop lesions upon inoculation of the cysts and the longest survival of amebae in these hosts was five days. When cysts were placed in conventional animals, 34 out of 37 animals developed amebiasis. Gnotobiotic animals monocontaminated with Escherichia coli or Aerobacter aerogenes developed amebiasis upon inoculation with the cysts.

In 1962 Coleman demonstrated the presence of bacteria within the rumen ciliate Entodinium caudatum, when, after sonication of the ciliate, about ten to one hundred times the amount of bacteria could be cultured than could be cultured prior to lysis of the protozoa. Two years later

Coleman (1964) presented a study in which Entodinium engulfed labelled bacteria. Escherichia coli was maximally engulfed at a rate of 200 bacteria/protozoan/minute and after 30 minutes only 12% of the engulfed bacteria remained viable. Fifty percent of the labelled bacterial carbon was retained by the protozoa and eighty percent of this fraction was demonstrated in newly synthesized protein. Coleman suggested that bacteria are important to the protozoa in: (1) lowering environmental redox potentials, (2) providing purines, pyrimidines and amino acids to protozoa in a situation where protozoa may require solid nucleic acid and protein particles to obtain sufficient amounts of the above materials, and (3) providing some low molecular weight compounds normally found either in the bacterial metabolic pool or as a bacterial enzyme.

The importance of bacteria in protozoan cultures as agents for lowering the redox potential was postulated by Jahn (1933). He said that the potential of medium alone could not be lowered as far as that shown in a rat cecum, even if the medium were under quite strict anaerobic conditions. If bacteria are added to the medium, then the potential could be lowered to that shown in the cecum. Johnson (1942) subjected bacteria-free cultures of Trichomonas vaginalis to aerobic and anaerobic conditions. He found reduced concentrations of organisms subjected to aerobic conditions as compared with those subjected to anaerobic conditions. It was also noted that a previous study by Trussell and Johnson (1941) showed the parasite incapable of utilizing carbohydrates under aerobic conditions but that it could do so in the presence of sodium thioglycollate.

Purification Methods for Histomonas and Other Protozoa:

Various methods have been employed by investigators to free other

species of protozoa from bacteria by washing or by washing combined with utilizing the organisms' migratory behavior. In 1928 A. K. Papart achieved bacteriological sterilization of Paramecium by successive washings with sterile water. It was stated that success of this procedure largely depended upon the organisms' abilities to migrate from the bacteria. Hetherington (1934) accomplished a similar method for Achromobacter inunctum by washing the organism several times in sterile medium and allowing the organisms to rapidly swim away from the bacteria. Again it was stated that, unless the organisms were rigorous at the onset, the method could not be depended upon to yield sterile protozoa. Purdy and Butterfield (1918) allowed planktonic organisms to actively migrate through a series of tubes containing sterile medium. Glaser and Coria (1930, 1935a) utilized the fact that their protozoa would migrate to the top of the tubes and separation of the protozoa from bacteria could be achieved through successive washings. Basically, all the methods utilizing washing as a method for isolation deal with negatively geotropic and motile properties of the protozoa for separation.

Another study by Glaser and Coria (1935b) utilized a V tube and semisolid gel as a means for purification of Tritrichomonas foetus. Using this method one arm of a V-shaped glass tube was filled with semisolid gel (agar base). A sample of the culture was then placed in the open arm of the V tube and protozoa allowed to migrate up through the gel. The incubation period varied from 16 to 22 hours at 18-22° C. When performed at 36° C most of the preparations were contaminated.

Another method utilized washing Tritrichomonas three to four times and then suspending the organisms on the surface of sterile saline in an upright tube ten feet long (Cleveland, 1928). The organisms were allowed

to free-fall with the idea that protozoa would fall faster than bacteria. Sterile fractions of protozoa were then collected from the bottom of the tube. Approximately half of the fractions collected were sterile, but when protozoa were inoculated into culture medium, they failed to grow unless bacteria were added to the inoculum. This same investigator tried washing three to four times by centrifugation until the ratio of protozoa to bacteria was approximately 50:1. He then took a loopful of this suspension containing 15-20 protozoa and inoculated into sterile medium. When tested 70-90% of the cultures appeared sterile but were later discovered to be contaminated because the bacteria did not grow at 36° C, the temperature used in testing for sterility.

Novy, et al. (1907) introduced three methods for purification of protozoa: (1) inoculating a culture sample above the fluid and allowing protozoa to migrate to the bottom of the tube (will not work if motile contaminants are present), (2) taking advantage of the marked aerotropism of trypanosomes by isolating bubbles of air around which these protozoa gather, and (3) smearing blood agar plates with a sample of culture fluid which formed colonies after 10-12 days at room temperature. Noguchi, et al. (1926) also isolated insect flagellates on blood agar plates around room temperature. He stated that it took approximately 7-10 days for colony formation.

Naegleria (Acanthamoeba) is a soil protozoan which has been studied with respect to its ability to transform between ameboid and flagellate states (Balamuth, 1965; Pittman, 1963; Willmer, 1963). The ameboid state has been interpreted as an adaptation for phagotrophy under conditions of available food. The flagellate stage, on the other hand, is an adaptation for fluctuating and unfavorable environmental conditions. Using

this principle cultures of the protozoa were established with single species of bacteria. If a culture sample were placed on a previously established monospecific bacterial layer, the protozoa would migrate radially onto the single species bacterial layer.

Previous studies have used antibiotics to attempt purification of protozoan cultures. As stated previously, Delappe (1953) used penicillin and streptomycin (100 or 150 units/ml) either singly or in combination to facilitate isolation of Histomonas meleagridis from a mixed bacterial culture; however, his attempts to free the cultures of bacteria were unsuccessful. He noted that if too great a decrease of the flora occurred, death of the flagellate ensued. In 1961 Lesser reported H. meleagridis being cultured successfully free of bacteria after treatment with antibiotics (4,000 units/ml penicillin, 40 mg/ml streptomycin, 200 units/ml mycostatin). Treated protozoa were placed in a medium containing fresh hamster liver but the protozoa could not be transferred more than ten consecutive times. In antibiotic studies with Entodinium caudatum, Coleman (1962) reported obtaining cultures that were almost bacteria-free but that such cultures could be maintained viable for only 3-4 days. The antibiotic concentration used in Coleman's study for purification was: 1695 units/ml penicillin, 4 mg/ml streptomycin sulfate, 4 mg/ml dihydrostreptomycin sulfate, and 4 mg/ml neomycin. This treatment generally resulted in cultures with approximately one bacterium per ten protozoa.

Another method used in obtaining pure cultures of protozoa has been by means of microisolation; however, this method generally is most reliable for isolating cysts or organisms whose population greatly exceeds that of the contaminant (Rees, et al., 1941; Jensen, 1951). Rees reported successful microisolation of Trichomonas foetus bacteria-free

(1937) and of Endamoeba histolytica cysts (1939). He found that T. foetus survived well without bacteria in culture. The isolated cysts of E. histolytica were cultured with a single species of bacteria. Jacobs (1947) micromanipulated cysts of E. histolytica and grew the protozoa with pure cultures of Clostridium perfringens. C. perfringens was selected because of the bacterium's susceptibility to penicillin, thus allowing easy elimination of the bacteria when necessary. When the cultures were subjected to 100-200 units/ml penicillin, survival and possible growth of the protozoa were indicated but their numbers began decreasing after 48 hours. Parallel to this culture, Jacobs used agar medium slants which were preconditioned by growing E. coli for 24 hours then heat killing the bacteria by subjecting the cultures to 56° C or 65° C for 2 hours. Heating did not kill all the bacteria but they appeared to be inhibited. Some cultures void of C. perfringens and grown on the preconditioned agar medium slants were maintained for 3.5 months, but after that time the cultures were suddenly lost. The reason for the loss was not apparent. Phillips, et al. (1955) also did micromanipulation studies with Endamoeba histolytica. They placed isolated protozoa into bacteria-free, conventional, and monoxenic birds. The bacteria-free birds did not produce lesions when infected. Conventional birds and monoxenic birds with Escherichia coli or Aerobacter aerogenes produced lesions.

Another approach for obtaining cultures of protozoa bacteria-free was by aseptically removing liver lesions from infected animals. Cleveland and Sanders (1930) tried establishing bacteria-free lesions of Entamoeba histolytica by infecting cats with the protozoan assuming the cat's liver capable of destroying many kinds of bacteria. Inoculation was done directly into the liver by hypodermic needle following laparotomy.

After lesions appeared, they were removed aseptically. Some lesions contained only bacteria, others only amebae. When the isolated lesions were placed in various media, none of the amebae survived longer than 14 days and those surviving did not multiply nearly as rapidly as those in cultures containing bacteria. Even when various substances were added to the medium, successful cultivation was not achieved; however, when the oxygen tension was reduced, some cultures appeared to have stimulated growth. Cauthen and Harris (1935) removed lesions from the breast muscle of a pigeon infected with Trichomonas columbae. Test for bacteria was done by making smears of lesions, staining them, and observing the slides microscopically. The authors placed the tissues on agar-medium slants and reported good growth after 24 hours incubation. After 5 days the tubes showed turbidity and gas formation. They attributed this to flagellate growth since no bacteria were observed microscopically using the hanging drop technique. Assuming liver lesions were bacteria-free, Miller (1951) cultured Entamoeba invadens on agar-medium slants. Harrison, et al. (1954) reported removing lesions aseptically from livers of turkeys infected with Histomonas meleagridis. A suspension of the lesions was injected into uninfected birds which subsequently developed lesions. These lesions showed no bacterial contamination. Bradley (1963) inoculated H. meleagridis obtained from lesions into avian embryos but the growth was highly variable.

MATERIALS AND METHODS

Cultivation of Histomonas meleagridis:

The original medium described by De Volt (1943) was made as follows: 9.0 g NaCl, 0.4 g KCl, 0.2 g $NaHCO_3$, 10.0 g glucose, 20.0 ml turkey serum and 1000 ml distilled water. In the present study, a slightly different formulation was used: 9.0 g NaCl, 0.4 g KCl, 0.2 g CaCl, 0.2 g $NaHCO_3$, 10.0 ml turkey serum, and 1000 ml double glass distilled, double deionized water. The glucose was removed and the serum concentration decreased to retard the growth of cecal bacteria which can initially outcompete the growth of histomonads. This modification was suggested by De Volt (1943) should bacterial growth become overwhelming using the original formulation.

In the preparation of the medium, the salts and bicarbonate were first dissolved in the water. The turkey serum was added and the pH of the medium adjusted to 9.0-9.2 using 0.1N NaOH. The pH adjustment prevents precipitation of serum proteins during autoclaving (De Volt, 1943). The medium was dispensed into screw-capped culture tubes with a Cornwall automatic pipette (5 ml per tube) and autoclaved at 15 lbs. pressure for 20 minutes. The tubes then can be stored in the refrigerator for approximately three months without affecting growth of cultures. Medium older than three months was not used since such medium resulted in variable protozoan growth (Larson, 1964; Ruff, 1968).

Blood from a normal turkey was obtained by cutting the jugular vein and dripping the blood into dry Petri dishes. Previous to use these plates were washed well with 7X detergent, rinsed several times with tap water, rinsed three times with distilled water and three times with deionized

cut into several pieces, and the Petri plate incubated at 35° C for 1 hour. The plates were then refrigerated overnight, the clots removed, and the serum centrifuged at 2000 rpm for 30 minutes to remove precipitated proteins. Sera showing evidence of extensive hemolysis were not used for culturing since Larson (1964) showed deleterious effects to the histomonads. The sera were stored in screw-capped vials at 0° C.

Water used in this laboratory for the medium was chemically purified as outlined by De Volt (1943). Distilled water from the building still was distilled twice through a glass distillation system and then passed twice through a deionizing column. This water was stored in a glass carboy until used. Apparently, there is an optimal purity of water for successful cultivation of histomonads. If the water was less pure, the degree of growth normally achieved in our laboratory was not accomplished. If the water was too pure (i.e., all metals removed), the parasites would not grow (Ruff, 1968).

In addition to liquid medium previously described, rice starch was added to cultures to provide an additional carbohydrate nutrient (De Volt, 1943). Powdered Bacto rice starch was found to be too coarse for efficient ingestion by histomonads so it was ground using a mortar and pestle for 40 minutes (Ostlind, 1966). Small quantities of the ground starch were placed into capped vials and sterilized by dry heat at 150° C for 90 minutes.

Ruff (1968) demonstrated that histomonad growth was more consistent if lumps of animal charcoal were added to cultures rather than charcoal powder used by De Volt (1943). These were prepared by placing lumps into capped vials and autoclaving for 20 minutes. Both starch and charcoal were added aseptically to culture tubes prior to inoculation.

Culture tubes and Pasteur pipettes used in cultivation procedures were acid-cleaned. The Pasteur pipettes were soaked in a sodium dichomate and sulfuric acid solution for 24 hours, rinsed in a pipette rinser for another 24 hours then rinsed twice in distilled water and twice in deionized water. When the pipettes were dry, they were placed in large, foil capped test tubes and autoclaved for 20 minutes. The culture tubes were soaked in concentrated nitric acid for 24 hours then rinsed ten times with tap water, three times with distilled water and two times with the water used for making the medium.

Another medium used in the laboratory was slightly modified Dwyer's medium (1970) which is considerably richer than the basic serum-saline medium of De Volt. The composition of this medium is: 85% M199, 10% normal horse serum and 5% chick embryo extract (McDougald, pers. comm.), and it differs from that of Dwyer (1970) in that Dwyer used 75% M199 instead of 85%. To this modified medium was added 10 mg powder rich starch per 10 ml medium. I found that if animal charcoal was added to the cultures, the growth and longevity of cultures were enhanced.

In Vitro Cultivation of Histomonas meleagridis:

Organisms used to begin an in vitro culture of histomonads were obtained from a turkey displaying overt symptoms of histomoniasis. Ten milliliters of modified De Volt's medium (39° C) was injected into an infected cecum and cecal contents withdrawn. A sample of the suspension was observed using a hemocytometer to verify the presence of histomonads. If histomonads were present, 0.25 ml of the suspension was inoculated into each of five culture tubes, each containing 5 ml medium. Approximately 4-5 mg of sterile ground rice starch and three lumps of animal charcoal

were added to each tube.

Subculturing established in vitro cultures was done approximately 48 hours after inoculation. The initial cultivation of cecal contents yielded high densities of bacteria that inhibited growth of the protozoa. Samples from each tube were examined in a hemocytometer to determine which tube had the greatest concentration of histomonads. From this tube 0.25 ml of suspension was inoculated into each of five tubes in the same manner as for the initial set of tubes. Subculturing was then done every fifth day in the manner described.

In Vivo Cultivation of Histomonas meleagridis:

Cultures of histomonads were maintained in turkeys since virulence of the protozoa regressed more slowly in vivo than in vitro. Two turkeys were initially infected with histomonads by administering 500-1000 embryonated eggs of Heterakis gallinarum known to harbor Histomonas meleagridis. The infection process was allowed to proceed until symptoms of histomoniasis were elicited.

One of the birds was sacrificed and the contents of the ceca removed by injecting 10 ml of De Volt's medium (39° C) into each cecum and withdrawing the suspension. Presence of histomonads was ascertained by microscopic examination. The suspension of cecal material was kept in a water bath at 39° C until two birds were ready for inoculation.

Two normal turkeys were prepared for inoculation by giving each an enema with 0.85% saline (39° C) to free the lower digestive tract of any fecal material that could block passage of histomonads into the ceca. After the birds defecated, 5 ml of the cecal suspension was administered to each bird through the cloaca. The birds were hanged by the feet for

20 minutes to allow the protozoa to reach the ceca. Subculturing was performed after symptoms of histomoniasis appeared.

Sucrose Density Gradient Centrifugation:

Nitrocellulose tubes (Beckman) designed for sucrose density gradient centrifugation were used. Since these tubes could not be autoclaved, they were sterilized by rinsing with 95% ethanol and exposing them for 48 hours to light in a UV chamber. Sterility of the tubes was tested at each trial by rinsing the inner portion of one of the tubes with medium and streaking the medium onto several trypticase soy agar plates. The plates were incubated aerobically and anaerobically at 37° C and at room temperature for about one week. Ultra violet light-ethanol proved effective for sterilization. The apparatus for collecting fractions from the tubes was similarly subjected to this method of sterilization and sterility checks.

Prior to determining the needed concentration gradients, the tolerance of the protozoa to the various concentrations of sucrose was determined. Sucrose concentrations ranging from 5% sucrose to 35% sucrose at 5% concentration intervals were used in the tolerance test. Conditions of the protozoa as determined by microscopic examination were recorded every 2 minutes for 30 minutes. Thirty minutes was selected as the end point for this test because the separation procedure could be completed within this time.

Density gradients were set up serially in the sterile nitrocellulose tubes using sterile sucrose solutions. The gradients ranged from 35% sucrose on the bottom to 5% sucrose on the top at 5% concentration intervals. Layering was accomplished by carefully pipetting each concentration

into the tubes. A culture sample containing 1×10^5 histomonads plus cecal bacteria was carefully layered on top of the 5% sucrose layer. Various centrifugation speeds and times were tested on an International, free-angle centrifuge. It was found that centrifugation for 10 minutes at 500 rpm proved optimal in obtaining the most protozoa without observable morphological effects.

Since it was thought that the volume of sucrose solution per layer could affect the degree of separation, two different volumes per sucrose layer were tested. Both 0.5 ml and 1.0 ml volumes of sucrose solution per layer were tested under the same centrifugation procedures to determine if better separation was achieved with the 1.0 ml per sucrose layer than with the 0.5 ml per sucrose layer. The 1.0 ml/layer was best for separating the protozoa from the bacteria.

After centrifugation the tube was placed into a sterile collecting apparatus and fractions were collected by puncturing the bottom of the tube. The five-drop samples were collected into sterile tubes and each sample was examined for histomonads and the samples were tested for sterility by plating on trypticase soy agar plates. These plates were incubated both aerobically and anaerobically for one week at 37° C and another set incubated at room temperature.

To determine the optimal concentration of histomonads required to start cultivation, different volumes of fractions containing histomonads were taken from the sucrose gradient and inoculated into tubes containing medium, sterile rice starch and animal charcoal. Some of the tubes were simultaneously inoculated with cultured cecal bacteria while other tubes were inoculated only with the histomonads. From the growth obtained, the optimum number of histomonads from a sucrose gradient were then cultured

with bacterial filtrate to see if cultivation of the histomonads could be accomplished using only bacterial by-products, thus void of viable bacteria.

Because the presence of sucrose in culture could be detrimental to growth of histomonads, another set of tubes received histomonads that were washed free of sucrose with sterile medium. Both this set of tubes and the set inoculated with unwashed histomonads were maintained in modified De Volt's medium (5 ml/tube) with the addition of 5 mg sterile rice starch and three lumps of sterile animal charcoal per culture tube. The cultures were examined for viable histomonads every day over a period of 20 days.

Isolation and Culturing of Lesions:

Since a number of investigators (Cleveland and Sanders, 1930; Cauthen and Harris, 1935; Miller, 1951; Harrison, et al., 1954; Bradley, 1963) have shown variable success in cultivation of various protozoa from excised liver lesions, this method was attempted as a possible method for the isolation of histomonads. It seemed conceivable that lesions containing only the protozoa and no bacteria could be isolated and cultured.

Turkeys inoculated with virulent histomonads were maintained until clinical symptoms of disease developed. The birds were then sacrificed and feathers removed from the sternal and abdominal regions. The exposed skin area of each bird was scrubbed well using 95% ethanol then a sterile cloth was placed over the body leaving the bare skin exposed. They were placed in a UV chamber in which the UV light had been on continuously for a minimum of 48 hours. The exposed skin was again scrubbed with 95% ethanol. Five different types of lesions on the liver were selected and each type removed separately using a different set of sterile instruments for each lesion. Each lesion was placed in a separate Petri dish containing

De Volt's medium. The lesion tissue was teased apart and suspended using sterile needles and Pasteur pipettes. When a good suspension was made, portions were used to test for bacterial contamination, to examine for histomonads and to inoculate directly into 6 tubes containing medium, sterile starch and charcoal. Three of the six tubes were incubated tightly capped in a 39° C water bath. The other three tubes were incubated aerobically at 39° C. Each of the five isolated liver lesions was treated in this manner resulting in a total of thirty tubes. Each tube was inspected for histomonads and tested for bacteria after 48 hours of incubation and weekly for 4 weeks.

A normal bird was also used to test the technique in isolating liver tissue. The same procedure was used for this bird as was used for the infected bird, and the tubes were maintained at the same time and under the same conditions as the cultures inoculated with liver lesion suspensions.

Selective Filtration with Millipore Filters:

Since it was suggested by several colleagues that protozoa could be selectively filtered from bacteria, a millipore filtration was attempted. Two porosities of filter were tested: 0.45 micron and 1.2 microns. The filters were set up in holders to be used with a vacuum pump. The entire set-up was autoclaved for 20 minutes at slow exhaust.

The millipore apparatus was placed in a sterile hood and attached to a vacuum pump. A 2 ml sample of an actively growing culture was placed on the filter surface and filtered at a vacuum of about 2-3 mm Hg. Just as the sample was pulled through, the sides and filter were rinsed with 10 ml sterile De Volt's medium. The rinsing procedure was repeated five

times. Care was taken to keep the filter as moist as possible during the operation. After the final rinse the apparatus was carefully disassembled and the filter carefully removed into a sterile Petri dish. The filter disc was then cut into four equal pieces using a sterile scalpel. One piece was tested for contamination by rubbing that piece over the surfaces of two trypticase soy agar plates, one of which was incubated aerobically and the other incubated anaerobically at 39° C. Each of the remaining filter pieces was placed into separate culture tubes containing De Volt's or Dwyer's medium, sterile rice starch and sterile charcoal. One set of tubes was tightly capped and incubated in a 39° C water bath while another set was incubated under anaerobic conditions at 39° C. All tubes were checked immediately after inoculation for the presence of viable histomonads. The tubes were checked again for histomonads 48 hours later and again after a week.

Antibiotic Treatment of Suspension Cultures:

Since the use of antibiotics showed variable success in obtaining protozoan cultures free of bacteria (Delappe, 1953; Lesser, 1961; Coleman, 1962), antibiotics were used in this phase of study to see if histomonads could be obtained bacteria-free. One particular antibiotic mixture was used extensively in this study. This was a mixture containing 20,000 units/ml penicillin, 20 mg/ml streptomycin and 1 mg/ml kanamycin in De Volt's medium.

In order to establish a range of effective antibiotic concentrations dilutions of the antibiotic mixture were made and a disc test performed. Small discs of filter paper were soaked in each dilution of a one to two dilution series from 1/2 to 1/1024. The discs were air dried in sterile

Petri dishes and placed on the surface of trypticase soy agar plates which had been previously smeared with an in vitro culture of cecal bacteria. Four discs were placed per plate and all tests were done in duplicate. The plates were then incubated aerobically and anaerobically at 39° C.

Once the effective range of concentrations was determined, these concentrations were tested on in vitro suspension cultures. The antibiotic mixture was added to the in vitro cultures to obtain final dilutions of 1/2, 1/4, 1/8 and 1/16. The protozoan cultures to which the antibiotics were added had been maintained in De Volt's medium until the protozoa has just broken lag and were in log growth phase before antibiotics were added. After the antibiotic mixture was added, the cultures were incubated in a water bath at 39° C in tubes with caps tightly screwed or in tubes which were placed in an anaerobic chamber at 39° C. After 24 hours incubation the cultures were tested for bacteria by plating a sample on trypticase soy agar plates which were incubated aerobically and anaerobically at 39° C for 96-120 hours. This test was run two separate times using two different cultures each time.

It was suggested that kanamycin might have a deleterious effect on the protozoa. A stock of the same antibiotic mixture was made up excluding kanamycin. The same test using the same conditions were run with this antibiotic mixture as with the previously described antibiotic mixture.

Since it was probable that De Volt's medium alone was insufficient to support the protozoa, a richer medium (Dwyer's) was also employed. Cultures in Dwyer's medium were started directly by inoculating from cultures established in De Volt's medium. The cultures were passed a

few times in Dwyer's medium before treatment with antibiotics. The original antibiotic mixture containing penicillin, streptomycin and kanamycin was used for this study. The cultures were subjected to the same antibiotic treatments as was done initially in this phase of investigation (1/2, 1/4, 1/8, 1/16 dilutions of antibiotic mixture). The cultures were incubated for 24 hours and after such time were inspected for histomonads and tested for bacteria anaerobically and aerobically on trypticase soy agar plates at 39° C.

Direct Agar Plating (Bacteriological Agar):

Another possibility for separating protozoa from bacteria was by propagating a culture on an agar surface. Novy, MacNeal and Torrey (1907) isolated colonies of mosquito trypanosomes on blood agar plates by smearing a series of plates, the last plate or two containing isolated colonies. Noguchi and Tilden (1926) isolated insect flagellates on blood agar plates and plant flagellates on leptospira media. Both the Novy study and the Noguchi study performed the operations around 18-28° C and incubated plates about 10 days for growth of colonies. Cultivation at room temperature provides a distinct advantage over cultivation at 39° C since bacteria will not flourish as well at room temperature. Histomonads, on the other hand, will not survive at lower temperatures and culturing must be done around 39° C. This particular technique was pursued since it provided some promise of success.

The agar media employed initially were standard bacteriological media: trypticase soy agar, blood agar, and thioglycollate agar (to provide a reducing agent should the protozoa require a low redox potential). A drop of an actively growing culture was smeared over plates with sterile

glass rods using a four-way streak (a different rod being used for each streak). The plates were then incubated at 39° C aerobically or anaerobically. Microscopic observations of the plates were made 72 hours post-inoculation and weekly thereafter for 4 weeks.

Bacterial Monolayer:

Naegleria is a soil protozoan which has been studied with respect to its ability to transform between ameboid and flagellate stages (Balamuth, 1965; Pittman, 1963; Willmer, 1963). Pittman (1963) stated that this protozoan was grown on agar surfaces with viable Klebsiella and also stated that attempts to grow the protozoan axenically were unsuccessful. The ameboid state of Naegleria has been interpreted to be an adaptation for phagotrophy under favorable food conditions. The flagellate stage, on the other hand, is an adaptation for fluctuating environmental conditions.

Using this principle it was possible to establish a protozoan culture with a single species of bacteria. If a compatible bacterial species could be found for the histomonads, then it was possible to establish the histomonads on a monospecific bacterial layer by placing a drop of the histomonad culture on a previously established layer of bacteria. The previously established single strain of bacteria should outcompete the bacteria present in the culture drop and if the bacterial species is compatible to growth of the histomonads, then the protozoa should slowly migrate into the monospecific bacterial population. Establishment of parasites with a single bacterial population would allow simpler procedures for isolation by means of antibiotics. Since histomonads display ameboid as well as flagellate properties, migrations are possible.

After reviewing papers on the microbial flora of turkeys (Harrison and Hansen, 1950) and on associations of histomonads with various strains of bacteria (Lesser, 1964; Bradley and Reid, 1966), the following bacterial strains were employed for study: Bacillus subtilis, Escherichia coli, Streptococcus faecalis, Lactobacillus sp., and Proteus mirabilis. The types of agar plates used were nutrient agar, trypticase soy agar, blood agar, thioglycollate agar and agar-media made with De Volt's medium or Dwyer's medium. Each strain of bacteria was plated onto each type of agar plate by distributing some bacterial suspension across the plates with sterile cotton swabs. One set of plates was incubated aerobically and another set incubated anaerobically at 39° C for 24 hours to set up a monobacterial layer. After incubation a drop of an actively growing parasite culture was placed on each monobacterial layer plate and incubated aerobically or anaerobically in a moist chamber at 39° C. Each plate was subjected to the same conditions as were control plates without the bacterial monolayer which received only one drop of culture. Each plate was examined microscopically for histomonads and bacteria at 0, 24, and 72 hours postinoculation. Plates were kept under above conditions and checked weekly for four weeks for evidence of growth.

De Volt's Agar Medium Plating:

Since microbiological agar media, blood agar in particular, did not support the cultivation of protozoa, a mixture of agar and a medium known to support growth of histomonads was tested. The first phase of this study involved the use of De Volt's medium with two different concentrations of agar, one at 1% and the other at 0.5% agar. The agar concentration was formulated for a softer surface than the standard 2% con-

centration used for bacteriological agar plates, because it was my belief that a softer surface would be more condusive to histomonad colony formation. When preparing these agar plates, the De Volt's medium was made, appropriate amounts of agar were added, and the mixture autoclaved at slow exhaust for 20 minutes. The 1% and 0.5% agar plates were used to plate histomonads directly onto the agar surface. Only the 0.5% agar was used to suspend organisms directly in the agar as well. Histomonads were suspended in the agar medium by suspending 0.25 ml of histomonad culture in 0.5% agar medium that had been equilibrated, after autoclaving, to 40° C in a water bath. After organisms were inoculated onto or into the agar medium, the plates were examined microscopically for presence of protozoa and then incubated aerobically or anaerobically in a moist chamber at 39° C. The plates were examined for histomonads after 24 and 72 hours post inoculation and weekly for 4 weeks.

Since successful establishment of histomonads could depend on additional nutrients than found in liquid De Volt's medium, various components were added singly or in combination to the 1% and 0.5% agar media. The factors that were altered were: (1) changing the serum concentration from 1% to 10%, (2) adding 2% glucose, (3) adding 5-10% bacterial filtrate, sonicated or heat-killed bacteria. The increased serum concentration allowed for added nutrients. The addition of 2% glucose was based on De Volt's original formulation in which he added 2% glucose to the medium for increased growth of histomonads. Addition of bacterial by-products might supply factors provided by bacteria which aid survival of the protozoa under more natural conditions.

Each factor was tested singly or in combination and at each agar concentration. Protozoa were streaked on the surface of the 1% and 0.5%

agar medium and inoculated within the 0.5% agar medium. Five plates were inoculated per set of conditions and were incubated aerobically or anaerobically at 39° C. The presence of histomonads was checked microscopically at 24 and 72 hours and weekly for 4 weeks. Another trial using the same procedures was executed at a later time using a different actively growing culture.

Dwyer's Agar Medium Plating:

Dwyer's medium is considerably richer than De Volt's medium and should provide any chemical nutrients required for maintenance of axenic histomonad cultures. For the agar medium in this study, agar concentration was reduced to 0.3% for an even softer agar. Three different concentrations of an antibiotic mixture added to the medium were tested for suppression of bacterial and fungal growth. The antibiotic mixture used contained 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin.

Culture samples were either streaked over the surface or suspended in the agar medium. The procedure followed was as outlined in the previous section. After inoculation, plates were examined for presence of histomonads and then incubated aerobically or anaerobically in a moist chamber at 39° C. The plates were subsequently observed microscopically after 24 and 72 hours postinoculation and weekly for 4 weeks.

The possibility of bacteria adding some component necessary for growth of histomonads was again tested by adding bacterial filtrate, bacterial sonicate or heat-killed bacteria. Since Dwyer's medium is so rich, I felt that nutrients other than some form of bacterial metabolite were not necessary to initiate growth of histomonads.

Agarose Medium Plating:

Agarose is a purified component of agar and generally results in plating efficiencies of tissue culture cells which are superior to efficiencies obtained using standard agar. I hoped that histomonads would respond in a similar manner.

In the first study a medium used for tissue culture was employed (RPMI 1640, Grand Island Biological Company). The agarose medium formulation used was 1640 + 20% fetal calf serum + 0.26% agarose + one of two antibiotic mixture concentrations. These concentrations were a 1/10 dilution and a 1/20 dilution of a stock antibiotic mixture described in the previous section. Fifteen milliliters of an actively growing culture of histomonads was obtained and centrifuged around 700 rpm for 8 minutes. The resulting pellet of histomonads was rinsed by resuspending with 15 ml of 1640 + 14.3% fetal calf serum + penicillin (200 units/ml) + streptomycin (0.2 mg/ml) and centrifuged at 700 rpm for 8 minutes. This rinsing procedure was repeated. The purpose of rinsing was to remove as much bacteria as possible. The pellet was resuspended in 2 ml of the above medium and counted. Appropriate dilutions were made so that approximately 10^2 or 10^3 histomonads could be plated per plate. These histomonads were plates alone or over feeder layers of human or mouse fibroblast monolayers using agarose medium. A control set of suspension cultures were run to determine if histomonads would grow in suspension using 1640 + 20% fetal calf serum + antibiotics. Five tubes each containing 5 ml of 1640 + 20% fetal calf serum + 10% antibiotic mixture and another set of tubes containing 1640 + 20% fetal calf serum + 5% antibiotic mixture were inoculated with the standard 0.25 ml inoculum. These tubes, as well as tubes not containing any antibiotics, did not produce cultures of suffi-

cient density for detection in a hemocytometer.

Human and mouse fibroblast monolayers were prepared the day before a plating. Actively growing confluent cells were used and the plates of monolayers were treated in the following manner: (1) medium in which the cells were growing was aspirated off, (2) the cells were gently rinsed with phosphate-buffered saline, (3) the saline was aspirated off, (4) 1 ml of 0.25% trypsin was placed onto the cells and the plates incubated at 37° C for about 10 minutes to loosen the cells, (5) loosened cells were then resuspended in 10 ml of MEM + 11% fetal calf serum (Grand Island Biological Company), (6) a few milliliters of the suspension were then diluted about 1/5 and 0.5 ml of the resulting suspension placed in Petri plates (60 ml), each containing 3.5 ml MEM + 11% fetal calf serum, and (7) plates were swirled well to distribute cells evenly and were incubated at 37° C in a CO_2 incubator for 24 hours.

In the second phase of this study, Dwyer's medium was used instead of the RPMI 1640 medium. Essentially the same procedure was followed in this phase, however, higher concentrations of the antibiotic mixture and a bacterial feeder layer in addition to human and mouse monolayers were used. The histomonads used in this phase had been grown for several passages in Dwyer's medium to equilibrate the culture prior to plating. The bacterial culture to be used as a feeder layer was started 24 hours prior to plating by drawing a sterile wire loop across an agar slant of Escherichia coli and plating the contents in 25 ml of Dwyer's medium. The inoculated medium was then incubated at 39° C until used the following day. E. coli was selected as the bacterial feeder layer species since studies using gnotobiotic birds have shown that histomonads in combination with E. coli have produced clinical symptoms of histomoniasis.

Agarose was added to the E. coli culture to be used as a feeder layer. The resulting concentration of agarose was 0.26%. Antibiotic mixture (20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin) was added to three different volumes of the agarose-bacteria medium in volumes sufficient to produce a final concentration ratio of 1/5, 1/8, or 1/10 with the medium. These antibiotic concentrations were equal to concentrations established in a subsequent layer of agarose medium to be layered upon the bacterial feeder layer. One milliliter of the agarose-bacteria medium was layered on the bottom of a Petri dish (60 mm) and allowed to set. Upon this feeder layer was layered 2.5 ml of a separating layer of agarose medium containing antibiotics equal in concentration to that of the feeder layer. This layer was allowed to set before proceeding. The final layer was agarose medium containing histomonads that had been washed twice with medium. The plates were incubated in moist chambers at 39° C. Prior to sealing the chambers, 10% CO_2 was flushed through the chamber for approximately 30 seconds to lower the amount of oxygen available in the system. The following day, plates were fed by gently adding to the surface 2 ml of Dwyer's medium containing the corresponding concentration of antibiotic mixture as in the plates. Plates were examined microscopically at this time for histomonads and bacteria. The plates were placed back into the chamber and flushed with CO_2 in the manner previously described. The chamber was opened and plates observed for histomonads and bacteria at one week intervals. Any plates exhibiting bacterial growth were discarded.

RESULTS

Sucrose Density Gradient Centrifugation:

After exposure of histomonads to various concentrations of sucrose to determine tolerance of the protozoa to the solutions, it was found that histomonads could withstand 15% and 20% sucrose solutions for thirty minutes. With higher sucrose concentrations (30% and 35%), however, changes in morphology of the organisms occurred after 16 minutes in 30% sucrose and after 14 minutes in 35% sucrose. The two latter sucrose concentrations are of greater significance because these concentrations were used to isolate histomonads by sucrose gradient technique. By the time the 10 minute centrifugation process was completed and fractions collected, sufficient time would have elapsed to stress the organisms enough to result in poor growth.

This particular method of separation was unreliable for obtaining sterile histomonads (Table 2). Two out of six trials (trials 1 and 4) yielded sterile histomonads and only the first two to three fractions were sterile. In those trials in which the first fraction was contaminated, the contamination appeared to be due to contaminated starch grains which centrifuged down with the histomonads.

To estimate the amount of inoculum to be used for starting cultures from a sucrose fraction, various numbers of histomonads from sterile fractions were taken and inoculated into tubes containing medium, sterile rice starch, animal charcoal and cecal bacteria cultivated in vitro. Viable histomonads were observed 48 hours after inoculation with 0.2 or 0.3 ml of the sucrose fraction containing histomonads (Table 3). From these results it was concluded that 0.25 ml of inoculum from the first

sucrose fraction should be used in subsequent tests.

Since the presence of sucrose in culture could adversely affect their growth by altering the tonicity of the medium or by changing the microbial populations (in those tubes containing microorganisms), two conditions were tested: (1) inocula of histomonads not washed free of sucrose and (2) *histomonads* washed free of sucrose with sterile medium. Table 4 shows that no histomonads could be cultured when inoculated only with bacterial filtrate. When the inoculum was accompanied by 0.25 ml of an actively growing *in vitro* culture of cecal bacteria, one out of five culture tubes from unwashed samples yielded histomonads after 24 hours cultivation but not thereafter. With the washed samples, again, no histomonads were recovered when cultured only with bacterial filtrate. When cultured with viable bacteria, histomonads were recovered 24 hours after inoculation but not thereafter. Attempts to subculture these organisms proved unsuccessful. Table 5 illustrates the standard growth of histomonads used in the experiment under normal subculturing procedures.

Isolation and Culturing from Lesions:

Table 6 shows that some tubes inoculated from liver lesion suspensions displayed growth of histomonads in De Volt's medium. The three tubes showing growth aerobically and the three showing growth anaerobically were inoculated with the same lesion suspension. That suspension also showed bacterial growth when plated on the initial day of inoculation, indicating the probable presence of bacteria in the liver lesion itself. Another set of tubes showed bacterial contamination after 48 hours incubation but not initially. This second set of tubes came from another

single liver lesion suspension. Apparently, the concentration of bacteria in that lesion was not high enough to be detected on the first day of isolation but after the liver suspension was cultured for a couple of days, bacterial numbers were sufficient to be detected.

Tubes with no bacterial growth had no histomonad growth. When all the lesion suspensions were microscopically examined, the concentration of protozoa present in each lesion suspension did not appear different. Thus, numbers of histomonads in lesions do not condition the success of in vitro cultivation. Since there seems to be a correlation between in vitro culture success of the protozoan and the presence of bacteria, it appears that the bacteria were important in aiding initiation of the histomonad culture. It was also noted that under anaerobic conditions those tubes showing protozoan growth established faster than tightly capped cultures. The presence of bacteria apparently is not necessary for establishment of protozoa, however, since viable protozoa were present in some liver lesions void of bacteria.

Except for one tube, all the control tubes with normal liver tissue were sterile. Since only one tube showed contamination, this contamination was probably introduced during sampling rather than from the tissue itself.

Selective Filtration with Millipore Filters:

Table 7 illustrates a summary of three separate trials at selective filtration. The histomonads thus obtained were cultivated in De Volt's medium. When the tubes were sampled initially for the presence of histomonads, viable and motile protozoa were observed microscopically. The 1.2 micron porosity filter was best for selectively filtering out the

bacteria from the culture sample. Although bacteria were present on the 0.45 micron filters, the concentration was low as demonstrated by minimal growth on the plates. The same types of results were obtained using Dwyer's medium (Table 8). The protozoa seemed to survive somewhat longer under anaerobic conditions with this medium when compared to De Volt's medium but the survival was still poor. What had not been previously anticipated in using this method was the presence of fungus in the cultures.

Apparently, fungus is ordinarily kept at low densities in cultures due to the presence of bacteria. When culture samples were filtered, fungal cells were retained on the filter as well as the histomonads because the fungal cells are approximately the same size as many histomonads. With a greatly diminished or eliminated bacterial flora the fungus flourished. After 48 hours incubation a large number of spherical bodies were evident in the cultures. Although these bodies did not appear as typical histomonads (typical histomonads were present), the spores were about the same size, so it was thought that these possibly were histomonads. It soon became evident, however, that these bodies were not histomonads, and were a fungus because hyphae began growing from these bodies.

Because the fungal cells are close to histomonads in size, this method was no longer considered feasible. This method was also not consistent for flushing the bacteria through the filters.

Antibiotic Treatment of Suspension Cultures:

Table 9 shows the results of the antibiotic disc test. The total inhibition zone defines that radial distance from the edge of the disc (in mm) showing no bacterial growth. The effective zone defines that radial distance from the edge of the disc (in mm) showing obvious deleter-

ious effects of the antibiotic on growth of bacteria. From these results, the first four dilutions of the antibiotic mixture were chosen to be used in the treatment study.

Table 10 shows that antibiotic treatment of histomonads to yield bacteria-free cultures was not successful using De Volt's medium. Although cultures demonstrated the presence of bacteria even at the 1/2 dilution level, the concentration of bacteria present in the tubes was low. The histomonad cultures could survive the 1/4 dilution treatment when placed in an anaerobic chamber but not when the tubes were just tightly capped. Apparently, low oxygen tension contributes to the establishment of the protozoa. Although the bacteria present in tightly capped cultures (1/4 antibiotic dilution) would establish anaerobic conditions eventually, it is possible that establishment is not rapid enough to facilitate growth of the protozoa.

Table 11 shows that removal of kanamycin from the antibiotic mixture resulted in an extensive dominance of fungal growth in the cultures. This antibiotic formulation was not considered further due to this complication.

The 1/2 and 1/4 dilutions of the stock antibiotic mixture in Dwyer's medium resulted in better survival of histomonads than in De Volt's medium (Figures 1 and 2), indicating that survival of protozoa may be nutritionally dependent or that the medium may help select for a particular bacterial species that better aids the protozoan's establishment. One observation, though casual, which was quite apparent in these cultures using Dwyer's medium was that the contamination present in the cultures was predominantly a chain-forming rod (generally forming very long chains). This was not observed in the antibiotic treated De Volt's cultures.

Dwyer's medium cultures treated with antibiotics also showed better

histomonad population growth than did the control cultures not containing any antibiotics (Figure 1). It appears that the use of Dwyer's medium provided the histomonads with some factor that better enables them to survive the decrease in bacterial density than can De Volt's medium. This is evidenced by poorer growth of histomonads in antibiotic treated De Volt's cultures when compared to untreated cultures (Figure 2). The protozoa may have really required some factor produced by the bacteria to survive in De Volt's medium; whereas, Dwyer's medium may provide nutritive factors that enable the protozoa to survive without multiplying bacteria. These observations suggest two hypotheses: (1) although the histomonads may require the presence of bacteria initially, after the protozoan culture establishes, the two populations may start to compete, and (2) the antibiotic may actually aid in selecting the dominance of a particular bacterial species (chain-forming rod) which is somehow contributing some factor better aiding establishment of the protozoan or which either does not or minimally competes with the protozoan. If the first situation is true, then bacteria indeed may not be required for establishment of protozoa; protozoa and bacteria may actually be competitors. Initially, it may be that bacteria set up some physical state that is condusive to establishment of the parasites, but bacteria may not be directly required for growth of the parasites. If the second situation is true, then the protozoan may actually require the presence of bacteria (possibly a particular type) or the protozoan may not be competing with the dominant strain.

Direct Agar Plating (Bacteriological Agar):

Table 13 illustrates the summary of results obtained from plating

the protozoan culture directly on microbiological agar surfaces. For each type of agar medium two sets of plates (5 plates per set) were incubated at 39° C, one set aerobically and one set anaerobically. Five separate series of plates were run (25 total plates) each employing the procedure stated in the previous statement.

Although viable histomonads were observable on the agar surfaces initially, colonies of the protozoa were not detectable at any time up to a month of observation. Bacteria were present in all plates. Although Novy, et al. (1907) and Noguchi and Tilden (1926) were able to establish isolated colonies of insect flagellates on blood agar plates, the histomonads did not plate under the conditions provided.

Bacterial Monolayer:

Table 14 shows results from microscopic examination of histomonads present on the surfaces of various agar plates inoculated with different strains of bacteria. Viable, active histomonads were observable at time 0 on the surface of the agar. Thereafter, however, no observable organisms could be detected for up to 4 weeks postinoculation. At an incubation temperature of 39° C it quickly became apparent that, in some cases, the bacteria were flourishing to the point of overgrowing and completely outcompeting the histomonads. In those plates where the bacteria did not grow, histomonads still did not grow. Some reasons which could account for this could be: (1) for some reason the histomonads will not grow on agar surfaces as will some other protozoa, (2) the protozoa may require the presence of bacteria for some factor necessary to growth, but too great a concentration of bacteria is detrimental due to physical and/or physiological limitations of the system, and (3) a mono-

specific bacterial system may be insufficient to support histomonads in vitro. Although single species of bacteria have been known to support histomonads in gnotobiotic turkeys, the in vitro system is not comparable to the in vivo system which has many factors that are unaccountable. In this phase of study it is, therefore, apparent that some factor(s) necessary for growth of histomonads was lacking.

De Volt's Agar Medium Plating:

Table 15 shows results obtained from microscopic observations of plates initially after inoculation, at 24 and 72 hours postinoculation, and weekly for 4 weeks. Although viable histomonads were visible on the surface upon inoculation, none were visible thereafter nor was there any evidence of colony formation. Presence of histomonads inoculated into the 0.5% agar medium was difficult to confirm microscopically, even initially after inoculation, due to the agar, so they were only presumed to be present initially. Bacteria grew in all plates and many plates displayed extensive growth by a week after inoculation.

Table 16 illustrates results from plates containing nutrients added singly or in combination with other nutrient factors. Although each set was tested aerobically (5 plates) and anaerobically (5 plates), the results were the same, so they were recorded as number of plates out of 10 total plates. Since two trials were performed resulting in the same results, the results were recorded as a summary of the two trials (i.e., 20 total plates).

Dwyer's Agar Medium Plating:

Table 17 shows results of platings of histomonad cultures with Dwyer's

trations of antibiotics and/or with a bacterial filtrate, bacterial sonicate or heat killed bacteria were negative for colonies of histomonads. Plates with 10% of the antibiotic mixture showed inhibited bacterial growth. Those with 25% of the mixture showed great inhibition and it appeared to be enough inhibition to possibly achieve successful isolation of colonies of histomonads. For the most part the plates with 50% of the antibiotic mixture were void of bacteria.

As a control for plating ability, the histomonads were inoculated into suspension cultures under the same conditions as the plates. One set was capped tightly as in the usual procedure and the other set placed under anaerobic conditions to keep the oxygen tension of the system low. It was observed that those tubes containing 10% or 25% antibiotics showed viable histomonads but those cultures with 50% antibiotics did not reveal histomonads during biweekly observations for up to 4 weeks. Those tubes receiving bacterial filtrate and heat-killed bacteria did not exhibit better growth than those not receiving any bacterial additive. Those receiving the bacterial sonicate did not do as well as the other tubes. The protozoa not growing in the 50% antibiotic mixture could be due to one or a combination of two reasons: (1) the antibiotic concentration was too high for the protozoa, and (2) the bacterial concentration in the culture was so low that some necessary factor, normally created by the bacteria, became deficient, thus hindering the establishment of the histomonads.

Agarose Medium Plating:

In the first phase of this study where 1640 + 20% fetal calf serum + 0.26% agarose + antibiotic mixture was used, some of the plates were

maintained bacteria-free but no microscopic evidence for colony formation or cloning was observed. Table 18 shows that a 1/20 dilution of the antibiotic mixture was not effective in obtaining bacteria-free plates. One plate with a 1/10 dilution of the antibiotic mixture was maintained bacteria-free for 4 weeks using a human fibroblast feeder layer. A number of what appeared to be viable histomonads were observed throughout the 4 week period but no mitoses were observed for any of the protozoa. Plates using mouse fibroblasts and feeder layers were discarded due to overgrowth of these cells causing contaminating growth of the mouse cells into the agarose layer containing the histomonads. The control set of suspension cultures in 1640 + 20% fetal calf serum + antibiotics showed no evidence of histomonad growth and showed a large amount of bacterial growth. Apparently, the medium itself would not support culture of the protozoa.

Table 19 shows results obtained from plating with Dwyer's agarose medium. Those plates using an Escherichia coli feeder layer were unsuccessful because the bacteria grew up and throughout the layer containing the histomonads. Those plates not using any bacterial feeder layer resulted in some plates that were bacteria-free. The plates that are recorded as having histomonads are plates that were bacteria-free; likewise, those that are recorded as not having histomonads contained bacteria.

After some time, the plate containing histomonads in the agarose medium with the 1/10 dilution of the antibiotic mixture showed contamination. Prior to the bacterial growth no mitoses were evident in the histomonads. The two plates with the 1/5 dilution of the antibiotic mixture remained sterile for a month but at no time were mitoses evident with the histomonads. The histomonads in the agarose medium, however, did

appear viable throughout. Apparently, the inability of the histomonads to mitose was not due to the antibiotic concentration since histomonads grew in suspension cultures containing the same concentrations of antibiotics. The suspension cultures all showed bacterial growth even with the highest concentration of antibiotics (1/5 dilution); yet, using the same concentration of antibiotics in the agarose medium two out of three plates were obtained bacteria-free.

DISCUSSION

Although attempts for axenic *in vitro* cultures of histomonads failed, further investigations of alternative methods may well be successful. Such studies would indicate the relationship these protozoa have with bacteria. They would aid in ascertaining factors that determine why one organism is pathogenic while another is not and if the type of bacterial flora influences pathogenicity.

Problems in obtaining axenic cultures have been attributed to a lack of understanding the symbiotic relationship between bacteria and histomonads. Previous studies of protozoan and bacterial relationships indicated that *Entodinium* (Bonhomme-Florentin, 1971) and *Crithidia* (Gill and Vogel, 1962) use bacteria as endosymbiotes. Bonhomme-Florentin (1971) suggested that the rumen ciliate *Entodinium*, which has an apparent obligate relationship with rumen bacteria, may utilize the bacteria for cellulose digestion. Gill and Vogel (1962) showed that bipolar bodies in *Crithidia* were bacterial endosymbiotes that synthesized lysine in the protozoan. Lee, et al. (1969) did not report any such relationship with *Histomonas meleagridis*.

Coleman (1962, 1964) presented results of work on *Entodinium* suggesting that these protozoa ingest and metabolize bacteria. He showed that the protozoa engulfed labelled bacteria (maximum was *Escherichia coli* at 200 bacteria/protozoan/minute) and that 40% of the labelled bacterial carbon was demonstrated in protein newly synthesized by the protozoan. Microscopic work by Lee, et al. (1969) on *H. meleagridis* indicated that no bacteria were present in the vacuoles of the protozoa. They also said that the histomonads appeared to be relying upon pinocytosis

rather than phagocytosis for nutrition.

One observation which appears to support the idea that the relationship between bacteria and histomonads is not obligatory is bacterially sterile lesions containing microscopically viable histomonads were isolated. Also, in the study using cultures suspended in antibiotics it was noted that histomonads in Dwyer's medium showed better populations when treated with antibiotics than when not. This was not true when De Volt's medium was used. An immediate conclusion one may draw is that Dwyer's medium supplies the histomonads with some factor(s) not present in De Volt's medium and that growing bacterial populations are not necessary for the maintenance of the protozoa. On further examination, however, two other possibilities should be considered.

In the first case, although the histomonads may require the presence of bacteria initially (for the establishment of some physical and/or physiological condition in the medium), they may actually compete with the bacterial population after the protozoan population establishes. If this is true, bacteria may not be required for maintenance of the protozoa. Lee et al. (1969) discovered that histomonads lack succinic dehydrogenase. Since this enzyme is part of the tricarboxylic pathway, they concluded that the histomonads are obligate anaerobes. If this were indeed the case, then bacteria may be required early to reduce the redox potential. This bacterial role in other protozoan cultures has been suggested by Jahn (1933). Such adjustments of the redox potential could be simulated.

In the second case, the antibiotics may selectively permit a dominant bacterial species (i.e., chain-forming rod) which somehow either contributes factor(s) aiding in establishment of the protozoa or which may not or minimally competes with the protozoa. This species may not grow

in De Volt's medium but may grow well in Dwyer's antibiotic treated medium. If this were true, one may conclude that either the protozoa truly require the presence of bacteria or that the protozoa simply are not competing with the dominant strain.

These possibilities and others exist and unanswered questions remain. Even when considering gnotobiotic studies where no clinical symptoms of disease were established without bacteria while clinical symptoms were established with certain types or combinations of bacteria, any of the above possibilities could be used to interpret the results.

On completion of this study some promising methods for obtaining axenic cultures became evident. Although some plates inoculated with histomonads using Dwyer's agarose medium containing antibiotics could be maintained without multiplying bacteria, using other methods for purification may be better to pursue. Such procedures would be: (1) a colloidal silica gel density gradient used by Wolff and Pertoft (1972) for the separation of HeLa cells in different stages of mitosis; (2) a serum gradient method used by Barbanti-Brodano, et al. (1973) for the separation of polykaryotic cells from normal cells; and (3) the use of an electrophoretic column using medium and different concentrations of Ficoll to establish a concentration gradient in combination with the electrical field.

Another possibly profitable approach to pursue would be to plate histomonads in agarose medium using turkey liver tissue as a feeder layer. Since sterile plates were obtained using antibiotics, a layer of liver tissue to feed the histomonads may provide some factor which could be utilized by the protozoa. Other things which could aid cultivation of axenic histomonads would be the addition of various amino acids, the use

of a good stable buffer such as Hepes to stabilize the pH of the cultures, and the addition of a good reducing compound.

SUMMARY

Sucrose Density Gradient Centrifugation:

Even though the sucrose density separation method yielded sterile fractions of histomonads: (1) the sucrose appeared to have an adverse effect upon the protozoa, (2) the protozoa could not be cultured without bacteria, and (3) cultured with bacteria the isolated protozoa still could not be recovered after 24 hours incubation.

Isolation and Culturing of Lesions:

The only lesions from which histomonads could be cultured in vitro were those which showed contamination with bacteria when tested on day 0. Those lesions which did not produce cultures of protozoa were those which either did not show bacterial contamination on day 0 or which did not show contamination when tested throughout the cultivation period.

Selective Filtration with Millipore Filters:

This method did trap the protozoa, however, at the same time fungal spores were also trapped on the filter. Upon cultivation the fungal spores flourished while the protozoa did not.

Antibiotic Treatment of Suspension Cultures:

The use of antibiotics did not prove successful in killing all the bacteria present in the in vitro cultures. Some interesting possibilities came about in this phase of study. When cultures were treated with antibiotics and cultured in Dwyer's medium (which is considerably richer in

nutrients than De Volt's medium), the histomonads grew at greater density than when not treated with antibiotics. Two possible inferences which could be drawn from this observation might be: (1) although the protozoa may require the presence of bacteria initially (for the establishment of some necessary factor), after the protozoan culture takes hold, it actually starts to compete with the bacterial population, or (2) the antibiotic may be selecting the dominance of a particular bacterial species (chain-forming rod not present in antibiotic-treated De Volt's cultures) which either is contributing some factor better aiding the establishment of the protozoa or which does not or minimally competes with the protozoa.

Plating Procedures:

Attempts to isolate cultures of protozoan cultures upon standard agar surfaces by streaking a culture drop were unsuccessful under aerobic and anaerobic conditions. The protozoa were observable after the streaking but not thereafter. Novy, et al. (1907) and Noguchi and Tilden (1926) established isolated colonies of an insect flagellate on blood agar plates but the histomonads did not plate under the conditions provided.

By taking advantage of the ameboid properties of this protozoan it was felt that protozoa in a culture drop placed centrally upon an agar surface, layered with a monospecific bacterial population, could migrate out onto the monospecific bacterial population. Possibly a culture of protozoa with a monospecific bacteria could be established and separation of the protozoa could be facilitated more easily than with a mixed bacterial culture. This was not successful under the conditions set for the histomonads since the plates had to be incubated at 39° C which allowed

bacteria to flourish and outgrow the protozoa.

An agar medium was formulated using De Volt's medium as the base for one medium and Dwyer's medium as the base for another medium. Different concentrations of agar were used but the protozoa could not be cultured either on the surface of the agar or suspended within the agar medium.

When histomonads were plated in Dwyer's medium with 0.26% agarose, what appeared to be viable protozoa were present but no mitoses were observed. A few plates were kept bacteria free for a month but mitosis of the protozoa was not observed. Apparently, the inability to mitose was not due to the antibiotic concentration since protozoa were able to grow in the same antibiotic concentration in suspension cultures.

TABLE I

Tolerance of Histomonads to Various Concentrations of Sucrose

Time (min.)	Sucrose Concentration (%)						
	5	10	15	20	25	30	35
2	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+
14	(+)	+	+	+	+	+	+
16	±	+	+	+	+	+	(+)
18	±	+	+	+	+	(+)	(+)
20	-	+	+	+	+	(+)	(+)
22	-	+	+	+	+	(+)	±
24	-	(+)	+	+	(+)	±	-
26	-	(+)	+	+	±	-	-
28	-	(+)	+	+	-	-	-
30	-	(+)	+	+	-	-	-

+ = healthy motile organisms
(+)= faded appearing organisms
± = membrane surface rough in appearance
- = no viable histomonads

TABLE II

Recovery of Histomonads and Bacteria in Various Fractions Collected from a Sucrose Gradient

Organism	Fraction Number	Trials 1	2	3	4	5	6
Histomonads	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	-	+	-	+	+
	4	-	-	-	-	-	-
	5	-	-	-	-	-	-
Bacteria	1	-	+	+	-	+	+
	2	-	+	+	-	+	+
	3	-	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+

+ = organism present
- = organism not detected

TABLE III

Cultivation of Histomonads from Various Inoculation Volumes from Sucrose Fractions Containing Isolated Histomonads

Tube Number	Inoculating Sucrose Volume (ml)[1/]				
	0.1	0.2	0.3	0.4	0.5
	Number of Histomonads/ml				
1	0	555	0	0	0
2	0	0	0	0	0
3	0	0	555	0	0
4	0	0	0	0	0
5	0	0	0	0	0

[1/] Gradient from which inocula was obtained contained 5×10^3 histomonads/ml

TABLE IV

Effects of Adding Bacterial Filtrate on the Growth of Sucrose Gradient Isolated Histomonads Unrinsed or Rinsed Free of Sucrose

Treatment	Tube Number	Bacterial Filtrate (%)						
		0	10	20	30	40	50	Control[1/]
		Number of Histomonads/ml						
Unrinsed Histomonads	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	555
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
Rinsed Histomonads	1	0	0	0	0	0	0	555
	2	0	0	0	0	0	0	1110
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0

[1/] 0.25 ml cecal bacteria/tube

TABLE V

Growth of Histomonads Using Standard Subculture Procedures

Tube Number	Time (days)						
	1	2	3	4	5	6	7
1	0	0	0	0.11	0.22	0.83	4.22
2	0.11	0.61	1.89	7.39	17.11	10.33	3.61
3	0	0.06	0.17	1.06	4.89	14.00	6.28
4	0.06	0.22	1.17	5.11	13.11	4.50	1.89
5	0	0	0.06	0.17	0.28	1.44	5.67

TABLE VI

Isolation of Histomonads and Bacteria from Liver Lesions or Uninfected Liver

Treatment	Length of Cultivation (days)	Infected Liver Lesions		Uninfected Liver
		Histomonads	Bacteria	Bacteria
		No. of Tubes Positive for Histomonads and Bacteria (out of 15)		
Capped	0	15	3	0
	2	0	6	0
	7	3	6	0
	14	1	6	0
	21	0	6	1
	28	0	6	1
Anaerobic	0	15	3	0
	2	3	6	0
	7	3	6	0
	14	0	6	0
	21	0	6	0
	28	0	6	0

TABLE VII

Cultivation of Histomonads in De Volt's Medium after Selective Filtration of a Mixed Culture

Filter Size (microns)	Treatment	Length of Cultivation (days)	Organisms Present		
			Histomonads	Bacteria	Fungus
			Number of Tubes Positive (out of 9)		
		0	9	9	nt[1/]
	Capped	2	3	9	9
		7	0	9	9
0.45					
		0	9	9	nt
	Anaerobic	2	0	9	9
		7	0	9	9
		0	9	0	nt
	Capped	2	0	0	9
		7	0	0	9
1.20					
		0	9	0	nt
	Anaerobic	2	0	0	9
		7	0	0	9

[1/] Organism was not observed microscopically and not specifically tested for

TABLE VIII

Cultivation of Histomonads in Dwyer's Medium after Selective Filtration of a Mixed Culture

Filter Size (microns)	Treatment	Length of Cultivation (days)	Organisms Present		
			Histomonads	Bacteria	Fungus
			Number of Tubes Positive (out of 9)		
		0	9	9	nt[1/]
	Capped	2	3	9	9
		7	0	9	9
0.45					
		0	9	9	nt
	Anaerobic	2	0	9	9
		7	0	9	9
		0	9	0	nt
	Capped	2	0	0	9
		7	0	0	9
1.20					
		0	9	0	nt
	Anaerobic	2	0	0	9
		7	0	0	9

[1/] Organism was not observed microscopically and not specifically tested for.

TABLE IX

Antibiotic Disc Test Against Cecal Bacteria on Trypticase Soy Agar Plates

Dilution[1]	Total Inhibition Zone[2] Distance from Disc (mm)		Antibiotic Effective Zone[3] Distance from Disc (mm)	
	Trial 1	Trial 2	Trial 1	Trial 2
1/2	7	8	11	10
1/4	6	7	11	10
1/8	6	6	10	9
1/16	5	5	10	9
1/32	3	3	10	8
1/64	1	2	7	6
1/128	0	0	6	5
1/256	0	0	6	5
1/512	0	0	5	4
1/1024	0	0	5	4

[1] Initial concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin.

[2] Radius from disc showing no bacterial growth.

[3] Radius from disc showing decreased bacterial growth.

TABLE X

Cultivation of Two Culture Lines (I and II) of Histomonads Treated with Antibiotics[1] in De Volt's

Treatment	Culture Line		Antibiotic Dilutions 1/2	1/4	1/8	1/16	Control Culture
			Number of Tubes Positive (out of 5)				
Anaerobic	Histomonas	I	0	1	4	5	5
		II	0	2	4	5	5
	Bacteria	I	4[2]	5	5	5	5
		II	5[2]	5	5	5	5
Capped	Histomonas	I	0	0	3	4	5
		II	0	0	4	4	4
	Bacteria	I	5[2]	5	5	5	5
		II	5[2]	5	5	5	5

[1] Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin

[2] Bacteria in low concentrations

TABLE XI

Cultivation of Two Culture Lines (I and II) of Histomonads Treated with Antibiotics[1] (without Kanamycin) in De Volt's Medium

Treatment	Culture Line		Antibiotic Dilutions 1/2	1/4	1/8	1/16	Control Culture
			Number of Tubes Positive (out of 5)				
Anaerobic	Histomonas	I	0	1	2	3	4
		II	0	0	1	1	5
	Bacteria	I	5[2]	5	5	5	5
		II	5[2]	5	5	5	5
	Fungus	I	4	5	3	3	0
		II	5	5	4	2	0
Capped	Histomonas	I	0	0	0	0	4
		II	0	0	0	1	4
	Bacteria	I	4[2]	5	5	5	5
		II	5[2]	5	5	5	5
	Fungus	I	5	5	4	4	0
		II	5	5	4	3	0

[1] Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin

[2] Bacteria in low concentrations

TABLE XII

Cultivation of Two Culture Lines (I and II) of Histomonads Treated with Antibiotics[1] in Dwyer's Medium

Treatment	Culture Line		Antibiotic Dilutions 1/2	1/4	1/8	1/16	Control Culture
			Number of Tubes Positive (out of 5)				
Anaerobic	Histomonas	I	1	4	5	5	5
		II	1	3	5	5	5
	Bacteria	I	5[2]	5	5	5	5
		II	5[2]	5	5	5	5
Capped	Histomonas	I	1	3	5	5	5
		II	0	3	4	5	5
	Bacteria	I	5[2]	5	5	5	5
		II	5[2]	5	5	5	5

[1] Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin

[2] Bacteria in low concentrations

TABLE XIII

Cultivation of Histomonads and Bacteria on Different Microbiological Agar Media under Aerobic and Anaerobic Conditions

	Media					
	Trypticase Soy		Blood		Thioglycollate	
Organisms	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
	Number of Plates Showing Growth (out of 25)					
Histomonads	0	0	0	0	0	0
Bacteria	25	25	25	25	25	25

TABLE XIV

Growth of Histomonads on Monobacterial Agar Plates

Length of Cultivation (days)	Bacterial Species	Histomonads	
		Aerobic	Anaerobic
0	Bacillus subtilis	+	+
	Escherichia coli	+	+
	Lactobacillus sp.	+	+
	Proteus mirabilis	+	+
	Streptococcus faecalis	+	+
1	B. subtilis	-	-
	E. coli	-	-
	L. sp.	-	-
	P. mirabilis	-	-
	S. faecalis	-	-
3	B. subtilis	-	-
	E. coli	-	-
	L. sp.	-	-
	P. mirabilis	-	-
	S. faecalis	-	-
21[1/]	B. subtilis	-	-
	E. coli	-	-
	L. sp.	-	-
	P. mirabilis	-	-
	S. faecalis	-	-

1/ Examined every 7 days up to 21 days.

TABLE XV

Effects of Agar Concentrations and Isolation Techniques on the Growth of Histomonads and Bacteria in De Volt's Agar Medium

	Culture Sample Streaked				Culture Sample Suspended	
	1% Agar Medium		0.5% Agar Medium		0.5% Agar Medium	
Organism	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
	Number of Plates Showing Growth (out of 5)					
Histomonads	0	0	0	0	0	0
Bacteria	5	5	5	5	5	5

TABLE XVI

Effects of Two Isolation Methods on the Subsequent Cultivation of Histomonads and Bacteria in De Volt's Agar Medium with Various Additives

Additives to Agar Medium	Culture Sample Streaked 1% Agar Medium		Culture Sample Suspended 0.5% Agar Medium	
	Histomonads	Bacteria	Histomonads	Bacteria
	Number of Plates Showing Growth (out of 20)			
0	0	20	0	20
10% Serum	0	20	0	20
10% Serum + 2% Glucose	0	20	0	20
10% Serum + 10% Bacterial Filtrate	0	20	0	20
10% Serum + 10% Bacterial Sonicate	0	20	0	20
10% Serum + 10% Heat-killed Bacteria	0	20	0	20
10% Serum + 2% Glucose + 10% Bacterial Filtrate	0	20	0	20
10% Serum + 2% Glucose + 10% Bacterial Sonicate	0	20	0	20
10% Serum + 2% Glucose + 10% Heat-killed Bacteria	0	20	0	20

TABLE XVII

Influence of Various Treatments of Dwyer's Agar Medium on the Growth of Histomonas meleagridis

Nutrient Additive	Antibiotic Additive[3]	Plates[1]				Suspension[2]	
		Histomonads		Bacteria		Histomonads	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Capped	Anaerobic
None	None	0	0	5	5	3	3
	10% Antibiotics	0	0	5	5	3	3
	25% Antibiotics	0	0	5	5	3	3
	50% Antibiotics	0	0	0	0	0	0
10% Bacterial Filtrate	None	0	0	5	5	2	3
	10% Antibiotics	0	0	5	5	3	3
	25% Antibiotics	0	0	5	5	3	3
	50% Antibiotics	0	0	0	1	0	0
10% Bacterial Sonicate	None	0	0	5	5	1	2
	10% Antibiotics	0	0	5	5	2	2
	25% Antibiotics	0	0	4	5	2	2
	50% Antibiotics	0	0	0	0	0	0
10% Heat-killed Bacteria	None	0	0	5	5	3	3
	10% Antibiotics	0	0	5	5	2	3
	25% Antibiotics	0	0	5	5	2	3
	50% Antibiotics	0	0	1	0	0	0

[1] 5 plates inoculated per treatment

[2] 3 tubes inoculated per treatment

[3] Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin

TABLE XVIII

Survival of Histomonads and Bacteria in 1640 + 20% Fetal Calf Serum + Antibiotics[1/] + 0.26% Agarose

	Plate Cultures		Suspension Cultures	
	Antibiotic Dilutions		Antibiotic Dilutions	
Organism	1/10	1/20	1/10	1/20
	Number of Plates (out of 3)		Number of Tubes (out of 5)	
Histomonads	1 (sterile)	0	0	0
Bacteria	2	3	5	5

[1/] Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, 1 mg/ml kanamycin

TABLE XIX

Survival of Histomonads and Bacteria in Dwyer's Medium + Antibiotics + 0.26% Agarose

Feeder Layer	Organism	Plate Cultures Antibiotic Dilutions 1/5	1/10	1/20	Suspension Cultures Antibiotic Dilutions 1/5	1/10	1/20
		Number of Plates (out of 3)			Number of Tubes (out of 3)		
Viable Cecal Bacteria	Histomonads	0	0	0	3	3	3
	Bacteria	3	3	3	3	3	3
None	Histomonads	2 (sterile)	1 (sterile)	0	3	3	3
	Bacteria	1	2	3	3	3	3

1/ Stock concentration: 20,000 units/ml penicillin, 20 mg/ml streptomycin, and 1 mg/ml kanamycin

Fig. 1. Growth of histomonads in Dwyer's medium treated with and without a 1/4 dilution of an antibiotic mixture containing penicillin, streptomycin and kanamycin.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

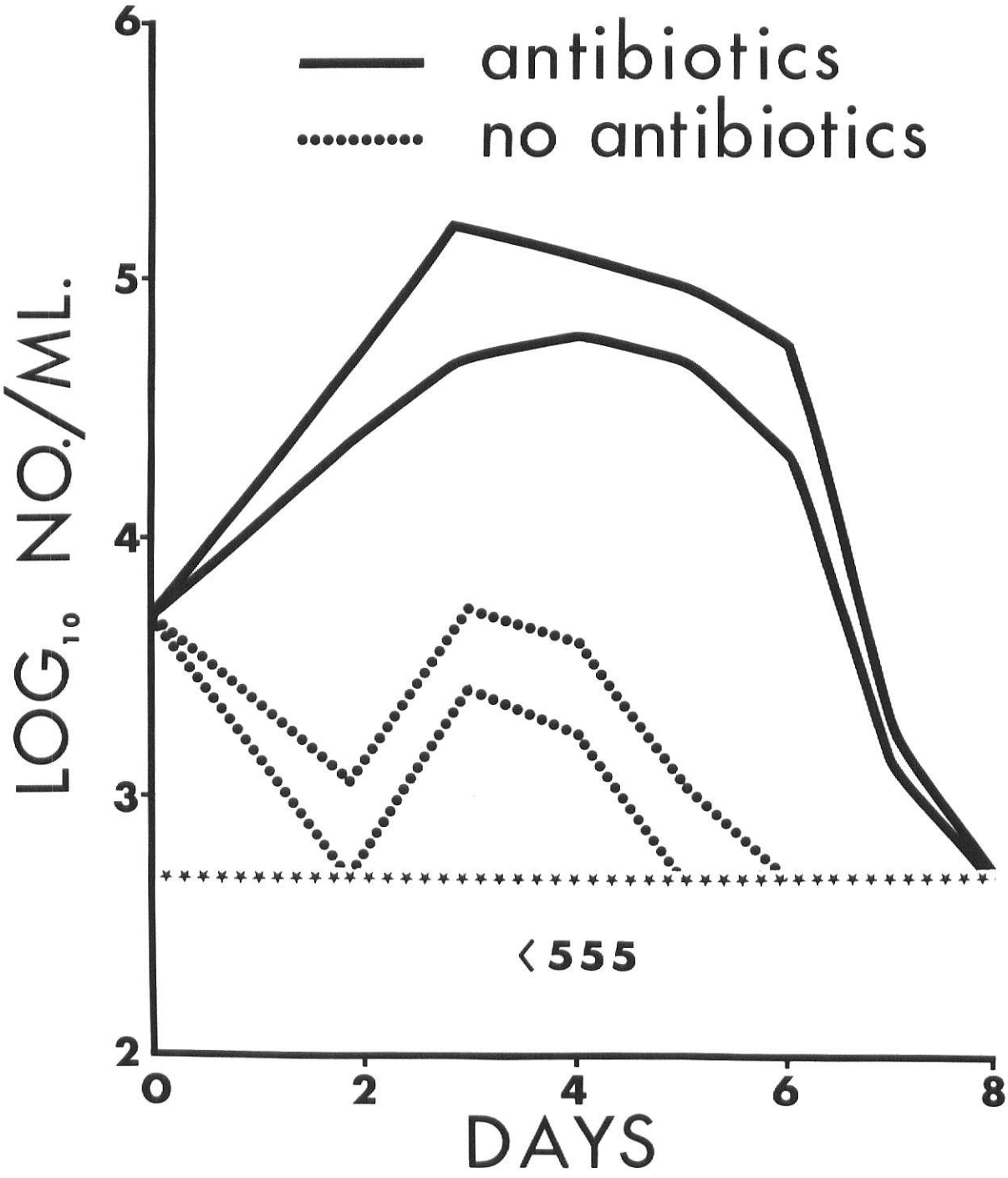
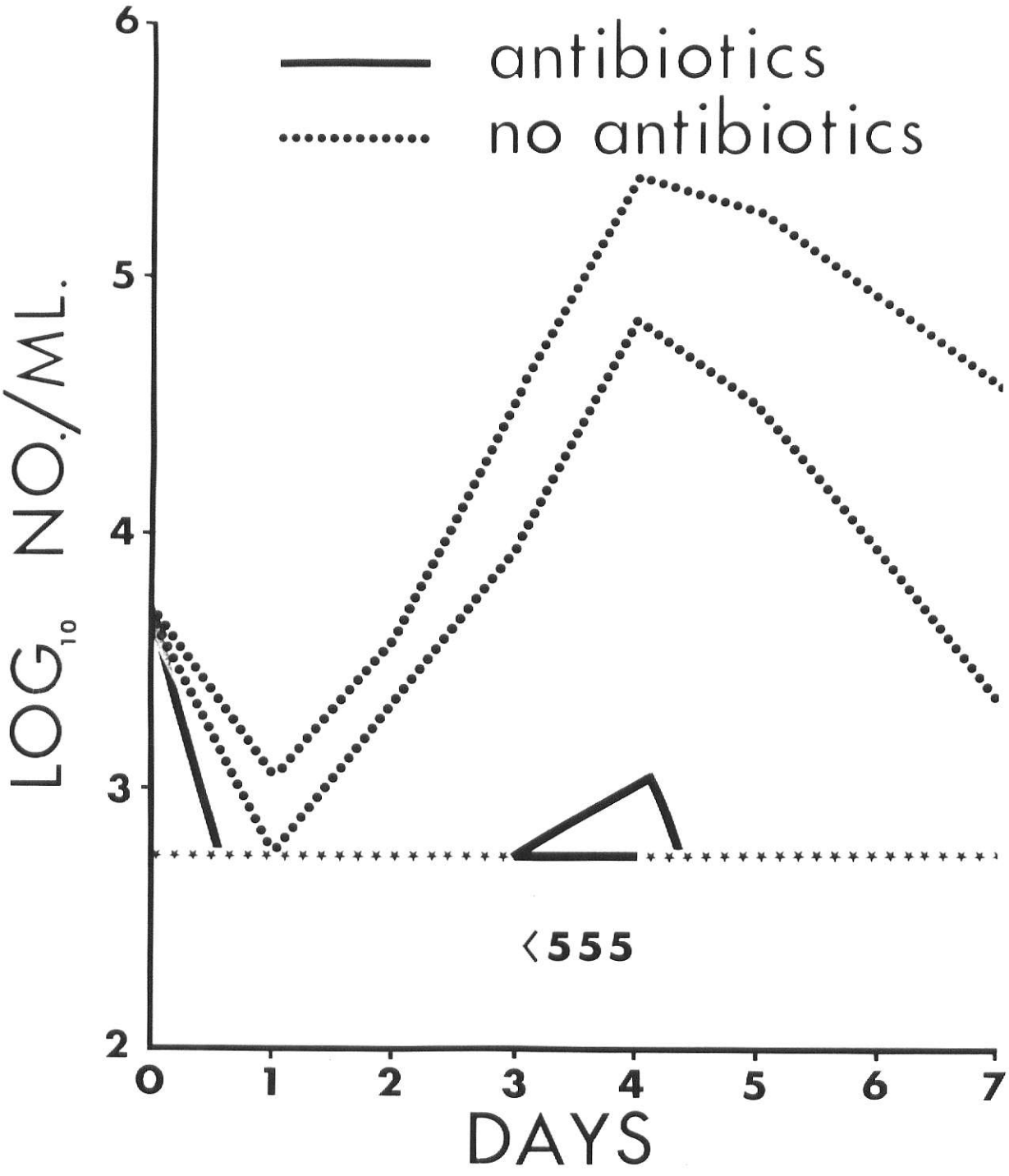

antibiotics
no antibiotics
LOG10 NO./ML.
6
5
4
3
2
0
2
4
6
8
DAYS
< 555

Fig. 2. Growth of histomonads in De Volt's medium treated with and without a 1/4 dilution of an antibiotic mixture containing penicillin, streptomycin and kanamycin


antibiotics
no antibiotics
LOG10 NO./ML.
6
5
4
3
2
0
1
2
3
4
5
6
7
DAYS
<555

ACKNOWLEDGEMENTS

The author wishes to express appreciation to Dr. M. F. Hansen, major professor, for his assistance and advice in experimental work and in the preparation of this thesis.

Appreciation is also expressed to other graduate students who aided in my work, especially to Dr. Ghassan Al-Khateeb. A special thanks to my husband Robert for his counsel and aid in my research and thesis.

REFERENCES

BALAMUTH, W. 1965. Amoebo-flagellate transformation as a model of reversible cyto-differentiation. In "Progress in Protozoology", p. 40. Second International Conference on Protozoology, London.

BARBANTI-BRODANO, G., POSSATI, L., MINELLI, G. P., AND LA PLACA, M. 1973. Use of a serum gradient for separation of polykaryotes from single cells after fusion with Sendai virus. Experimental Cell Research 78, 376-382.

BISHOP, A. 1938. Histomonas meleagridis in domestic fowls (Gallus gallus). Cultivation and experimental infection. Parasitology 30, 181-194.

BOECK, W. C., AND DRBOHLAV, J. 1925. The cultivation of Endamoeba histolytica. American Journal of Hygiene 5, 371-407.

BONHOMME-FLORENTIN, A. 1971. Oligotrichous ciliates, rumen bacteria and cellulose digestion. Protistologica 6, 383-388.

BRADLEY, R. E. 1963. Growth of Histomonas meleagridis (Smith, 1895) in avian embryos. Journal of Parasitology 49 (supplement), 62.

BRADLEY, R. E., JOHNSON, J., AND REID, W. M. 1965. Apparent obligate relationship between Histomonas meleagridis and Escherichia coli in producing disease (in turkeys). Journal of Parasitology 50 (supplement), 51.

BRADLEY, R.E., AND REID, W. M. 1966. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. Experimental Parasitology 19, 91-101.

CAUTHEN, G. E., AND HARRIS, M. M. 1935. The cultivation of Trichomonas columbae free from bacteria. American Journal of Hygiene 22, 364-365.

CLEVELAND, L. R. 1928. The suitability of various bacteria, molds, yeasts and spirochaetes as food for the flagellate Tritrichomonas fecalis of man, etc. American Journal of Hygiene 8, 990-1013.

CLEVELAND, L. R., AND SANDERS, E. P. 1930. The production of bacteria-free amoebic abscesses in the liver of cats and observations on the amoebae in various media with and without bacteria. Science 72, 149-151.

COLEMAN, G. S. 1962. The preparation and survival of almost bacteria-free suspensions of Entodinium caudatum. Journal of General Microbiology 28, 271-281.

COLEMAN, G. S. 1964. The metabolism of Escherichia coli and other bacteria by Entodinium caudatum. Journal of General Microbiology 37, 209-223.

DELAPPE, I. P. 1953. Studies on Histomonas meleagridis. I. Use of antibiotics to facilitate in vitro isolation. Experimental Parasitology 2, 79-86.

DE VOLT, H. M. 1943. A new medium for the cultivation of Histomonas meleagridis. Journal of Parasitology 29, 353-355.

DE VOLT, H. M., AND DAVIS, C. R. 1936. Blackhead (infectious enterohepatitis) in turkeys with notes on other intestinal protozoa. Maryland Agricultural Experiment Station Bulletin, No. 392, 493-567.

DOBELL, C., AND LAIDLAW, P. P. 1926. On the cultivation of Entamoeba histolytica and some other entozoic amoeba. Parasitology 18, 283-318.

DOLL, J. P., TREXLER, P. C., REYNOLDS, L. I., AND BERNARD, G. R. 1963. The use of peracetic acid to obtain germfree invertebrate eggs for gnotobiotic studies. American Midland Naturalist 69, 231-239.

DRBOHLAV, J. 1924. The cultivation of the protozoan of blackhead. Journal of Medical Research 44, 677-681.

DWYER, D. M. 1970. An improved method for culturing Histomonas meleagridis. Journal of Parasitology 56, 191-192.

FARMER, R. K., AND STEPHENSON, J. 1949. Infectious enterohepatitis (blackhead) in turkeys: A comparative study of methods of infection. Journal of Comparative Pathology and Therapeutics 59, 119-126.

FARR, M. M. 1956. Survival of the protozoan parasite, Histomonas meleagridis, in feces of infected birds. Cornell Vet. 46, 178-187.

FARR, M. M. 1961. Further observations on survival of the protozoan parasite, Histomonas meleagridis, and eggs of poultry nematodes in feces of infected birds. Cornell Vet. 51, 3-13.

FRANKER, C. K., AND DOLL, J. P. 1964. Experimental histomoniasis in gnotobiotic turkeys. II. Effects of some cecal bacteria on pathogenesis. Journal of Parasitology 50, 636-640.

GIBBS, BYRON JUNIUS. 1958. A study of Histomonas meleagridis, a protozoal parasite transmitted by a nematode. Ph.D. Thesis, University of Minnesota.

GIBBS, B. J. 1962. The occurrence of the protozoan parasite Histomonas meleagridis in the adults and eggs of the cecal worm Heterakis gallinae. Journal of Protozoology 9, 288-293.

GILL, J. W., AND VOGEL, H. J. 1962. Lysine synthesis phylogeny: biochemical evidence for bacterial type endosymbiote in the protozoan Herpetomonas (Strigomonas) oncopelti. Biochimica et Biophysica Acta 56, 200-201.

GLASER, R. W., AND CORIA, N. A. 1930. Methods for the pure culture of certain protozoa. Journal of Experimental Medicine 51, 787-806.

GLASER, R. W., AND CORIA, N. A. 1935a. The culture and reactions of purified protozoa. American Journal of Hygiene 21, 111-120.

GLASER, R. W., AND CORIA, N. A. 1935b. Purification and culture of Tritrichomonas foetus (Riedmüller) from cows. American Journal of Hygiene 22, 221-226.

GRAYBILL, H. W., AND SMITH, T. 1920. Production of fatal blackhead in turkeys by feeding embryonated eggs of Heterakis papillosa. Journal of Experimental Medicine 31, 647-655.

HARRISON, A. P., JR., AND HANSEN, P. A. 1950. The bacterial flora of the cecal feces of healthy turkeys. Journal of Bacteriology 59, 197-210.

HARRISON, A. P., JR., HANSEN, P. A., DE VOLT, H. M., HOLST, A. P., AND TROMBA, F. G. 1951. Studies on the pathogenesis of infectious enterohepatitis (blackhead) of turkeys. Poultry Science 33, 84-93.

HETHERINGTON, A. 1934. The sterilization of protozoa. Biological Bulletin 67, 315-321.

HORTON-SMITH, C., AND LONG, P. L. 1956. Studies on histomoniasis. I. The infection of chickens (Gallus gallus) with histomonad suspensions. Parasitology 46, 79-90.

JACOBS, L. 1947. The elimination of viable bacteria from cultures of E. histolytica and the subsequent maintenance of such cultures. American Journal of Hygiene 46, 172-176.

JAHN, T. L. 1933. Studies on the oxidation-reduction potential of protozoan cultures. I. The effect of pH on Chilomonas paramecium. Protoplasma 20, 90-104.

JENSEN, W. I. 1951. A study of Histomonas meleagridis in culture and in the tissues of the host. M.S. Thesis, Cornell University.

JOHNSON, J. G. 1942. Physiology of a bacteria-free culture of Trichomonas vaginalis. IV. Effect of hydrogen ion concentration and oxygen tension on population. Journal of Parasitology 28, 369-379.

LARSON, I. 1964. Biological aspects of enterohepatitis in fowl relative to Histomonas meleagridis and Heterakis gallinarum. Ph.D. Thesis, Kansas State University, Manhattan.

LEE, D. L., LONG, P. L., MILLARD, B. J., AND BRADLEY, J. 1969. The fine structure and method of feeding of the tissue parasitizing stages of Histomonas meleagridis. Parasitology 59, 171-184.

LESSER, E. 1960. Cultivation of Histomonas meleagridis in a modified tissue culture medium. Journal of Parasitology 46, 686.

LESSER, E. 1961. In vitro cultivation of Histomonas meleagridis free of demonstrable bacteria. Journal of Protozoology 8, 228-230.

LESSER, E. 1964a. Studies on the in vitro cultivation of Histomonas meleagridis with three different mixed bacterial flora. Proceedings of the Helminthological Society of Washington 31, 263-264.

LESSER, E. 1964b. Studies on the in vitro growth of Histomonas meleagridis with single species of bacteria. Proceedings of the Helminthological Society of Washington 31, 265-266.

LUND, E. E., AUGUSTINE, P. C., AND ELLIS, D. J. 1966. Immunizing action of in vitro attenuated Histomonas meleagridis in chickens and turkeys. Experimental Parasitology 18, 403-407.

MC DOUGALD, L. R. pers. comm. Senior Scientist, Eli Lilly and Company, Greenfield, Indiana, 46140.

MILLER, M. J. 1951. A method for in vitro culture of Entamoeba invadens free from bacteria. Canadian Journal of Comparative Medicine 15, 268.

NOGUCHI, H., AND TILDEN, E. B. 1926. Comparative studies of Herpetomonads and Leishmanias. I. Cultivation of Herpetomonads from insects plants. Journal of Experimental Medicine 44, 307-326.

NOVY, F. J., MAC NEAL, W. J., AND TORREY, H. N. 1907. The trypanosomes of mosquitos and other insects. Journal of Infectious Diseases 4, 223-276.

OSTLIND, D. 1966. Effects of x-rays on the developmental biology of Heterakis gallinarum and on the pathogenicity of Histomonas meleagridis. Ph.D. Thesis, Kansas State University, Manhattan.

PARPART, A. K. 1928. The bacterial sterilization of Paramecium. Biological Bulletin 55, 113-120.

PHILLIPS, B. P., WOLFE, P. A., REES, C. W., GORDON, H. A., WRIGHT, W. H., AND REYNIERS, J. A. 1955. Studies on the ameba-bacteria relationship in amebiasis. Comparative results of the intracecal inoculation of germfree, monocontaminated, and conventional guinea pigs with Entamoeba histolytica. American Journal of Tropical Medicine and Hygiene 4, 675-692.

PITTMAN, M. 1963. Studies of an amoebo-flagellate Naegleria gruberi. Quarterly Journal of Microscopical Science 104, 513-529.

PURDY, W. C., AND BUTTERFIELD, C. T. 1918. Effect of plankton upon bacterial death rates. American Journal of Public Health 8, 499-505.

PYNE, C. K. 1961. Etude de la structure inframicroscopique de Strigomonas oncopelti (Flagelle, Famille Trypanosomidae). In "Progress in Protozoology", p. 396. Second International Conference on Protozoology, London.

REES, C. W. 1937. Obtaining bacteria-free pure lines of Trichomonas foetus by means of microisolation. American Journal of Hygiene 26, 283-291.

REES, C. W. 1939. The microisolation of Endamoeba histolytica and its cultivation with single species of bacteria. Journal of Parasitology (supplement) 25, 14.

REES, C. W., REARDON, L. V., AND JACOBS, L. 1941. The cultivation of the parasitic protozoa without bacteria. American Journal of Tropical Medicine 21, 695-716.

RUFF, M. D. 1968. Effects of gamma radiation on in vitro growth on in vivo pathogenicity of *Histomonas meleagridis*. *Ph.D. Thesis*, Kansas State University, Manhattan.

SPRINGER, W. T., JOHNSON, J., AND REID, W. M. 1969. Transmission of histomoniasis with male Heterakis gallinarum (Nematoda). Parasitology 59, 401-405.

TRUSSELL, R. E., AND JOHNSON, G. 1941. Physiology of a pure culture of Trichomonas vaginalis. III. Fermentation of carbohydrates and related compounds. Proceedings of the Society for Experimental Biology and Medicine 47, 176-178.

TYZZER, E. E. 1919. Developmental phases of the protozoan of "Blackhead" in turkeys. Journal of Medical Research 40, 1-30.

TYZZER, E. E. 1934. Studies on histomoniasis or "blackhead" infection in the chicken and the turkey. Proceedings of the American Academy of Arts and Sciences 69, 189-264.

TYZZER, E.E., AND COLLIER, J. 1925. Induced and natural transmission of blackhead in the absence of Heterakis. Journal of Infectious Diseases 37, 265-276.

TYZZER, E. E., AND FABYAN, M. 1921. Further studies on "Blackhead" in turkeys with special reference to transmission by inoculation. Journal of Infectious Diseases 27, 207-223.

WILLMER, E. 1963. Differentiation in Naegleria. Symposia of Society for Experimental Biology 17, 215-233.

WOLFF, D. A., AND PERTOFT, H. 1972. Separation of HeLa cells by colloidal silica density gradient centrifugation. I. Separation and partial synchrony of mitotic cells. Journal of Cell Biology 55, 579-585.

ATTEMPTS AT AXENIC CULTIVATION OF *HISTOMONAS MELEAGRIDIS*

by

DIANE S. HIRSCH

B.A., University of Hawaii, Honolulu, Hawaii, 1970

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Parasitology

DIVISION OF BIOLOGY

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1975

Although a previous investigator (Lesser, 1961) reported successful axenic cultures of Histomonas meleagridis, attempts by this investigator to duplicate his results failed. Alternative methods, therefore, were tried for purification and in vitro cultivation of histomonads. Methods which did not yield bacteria-free histomonads were selective filtration using millipore filters, antibiotic treatment of suspension cultures, and plating the histomonads on the surface or suspended within bacteriological agar, De Volt's agar medium, and Dwyer's agar medium. Sucrose density gradient centrifugation, isolation of sterile liver lesions from infected birds, and plating in an agarose medium with antibiotics resulted in pure histomonads, but successful cultivation of the parasite was not achieved using these methods.