FACTORS AFFECTING THE IN VITRO CULTIVATION OF HISTOMONAS MELEAGRIDIS

by

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INTRODUCTION

Successful *in vitro* cultivation of an experimental organism is a basic and important step in many fields of experimental biological research. Of primary concern is a culture medium which yields abundant and consistent growth of an organism without attenuation. The success or failure of the various media for growth of *Histomonas meleagridis* is well documented in the literature; however, information is lacking on factors influencing multiplication of this organism.

The current study is concerned with factors influencing the growth of *H. meleagridis* in a modified DeVolt's alkaline serum medium (DeVolt, 1943). This medium was selected because it has the characteristics of ease of maintenance, favors good growth and longevity of the protozoan. Factors studied included agitation of the tubes, optimum quantities of starch and charcoal, oxygen tension, age and method of preservation of turkey serum, age of culture, refrigeration, freezing and effect of a freezing preservative (glycerol).

The purpose of this study was to determine the combination of the before mentioned factors which, giving the best multiplication and longevity of the organism, would also provide a uniformity of multiplication between cultures. Cryobiologic studies are of interest because successful freezing of the organism would permit the preserving of cultures for long periods, thereby maintaining organisms of a given genetic strain.

LITERATURE REVIEW

Several media have been used with varying degrees of success in culturing Histomonas meleagridis. Drbohlav (1924) successfully maintained the organism on slants of coagulated egg white covered with blood bouillon containing one per cent peptone. Tyzzer (1934) obtained good growth in a medium composed of buffered egg albumen agar slant covered with five per cent sterile horse serum in saline. Bishop (1938) successfully cultured the organism from liver lesions using Dobell and Laidlaw's inspissated horse serum diluted 1:8. DeVolt and Davis (1936) used Locke-egg-serum medium with Locke's solution containing turkey serum or a combination of serum and albumen. DeVolt (1943) obtained luxuriant growth on a new medium composed of Locke's solution containing two per cent turkey serum and two per cent of 0.2N sodium hydroxide. Since unprecipitated serum supported more growth than precipitated serum, sodium hydroxide was added to prevent precipitation of serum protein upon autoclaving. Rice starch was added to the medium before inoculating with histomonads.

Various methods have been suggested for continued maintenance of the organism in culture. Delappe (1953a) used penicillin and streptomycin in Laidlaw's medium to facilitate in vitro isolation of the organism. Lesser (1960) cultured the organism without viable bacteria in tissue culture media using Selas filtered cream, antibiotic treated nutrient broth culture of turkey cecal bacteria, and penicillin G potassium, dehydrostreptomycin sulfate, and mycostatin. Lesser (1961) also cultured H. meleagridis in vitro axenically using a modified culture medium enriched by fresh hamster liver tissue and metallic ions. He also had success with hamster kidney tissue (Lesser, 1963a).

Bradley (1963) successfully grew the organism in avian embryos.

Lesser (1963b) found that the Amphotericin-B inhibited the growth of histomonads in cultures containing cholesteryl palmitate or cholesteryl stearate, but not in cultures containing cream. Concerning the relationship between various bacteria and the growth of the organism, he (1964a) found that pure cultures of Escherichia coli, E. freundii, Proteus misabilis and a lactobacillis failed to support the in vitro growth of Histomonas, and (1964b) that enteric bacteria from hamsters, gerbils, and chickens were not as effective as turkey cecal bacteria for supporting the in vitro growth of H. meleagridis.

Larson (1964) was unsuccessful in culturing H. meleagridis in Tyzzer's, Laidlaw's and Delappe's mediums, but was able to grow the organism through 56 transfers covering 11 months in DeVolt's medium (without glucose). Ostlind (1966) cultured the organism successfully using a modified DeVolt's medium. He reduced the turkey serum to 15 ml (1.5%) and used double distilled water at a pH 9.25-9.50. Ruff (1968) further modified DeVolt's medium by using double glass distilled, deionized water, 10 ml (1.0%) turkey serum at a pH of 9.0-9.25.

Ostlind (1966) found that 0.5 g of finely ground Bacto rice powder per tube of medium and an undefined amount of ground animal charcoal increased the longevity of cultures. Ruff (1968) used lump charcoal to facilitate easier counts and better multiplication of the organism.

Larson (1964) reported that a Levy hemacytometer with Neubauer ruling provided a means for accurately determining numbers of H. meleagridis in culture media. To obtain an enumeration of a population, a tube was agitated to distribute the histomonads after which samples were taken with a Pasteur pipette and used to fill both chambers of the hemacytometer. Six counts were made per tube. Ostlind (1966) developed a method utilizing this technique for determining the number of organisms per milliliter.

The organism has been reported to grow both aerobically and anaerobically. Delappe (1953b) reported good anaerobic growth of Histomonas in the presence of cysteine HCl (0.1%) as a reducing agent and characterized the organism as being a facultative anaerobe. Lesser (1960b) obtained an anaerobic state by overlaying each culture with 0.5 ml white petroleum jelly which was liquified in a flamed hypodermic syringe. He found a pH of 5.8–6.8 best under these conditions.

Protozoa have been frozen successfully in dimethylsulfoxide (DMSO) and in glycerol; however, none has been reported to have survived without preservative. The author was unable to find any literature dealing with the freezing of Histomonas meleagridis.

One of the earliest reports citing the use of cryobiologic techniques for preserving protozoans was made by Coggeshall (1939) in which he reported successful preservation of malarial parasites by freezing. Little advance was made in the field of cryobiology for 20 years.

Diamond, Meryman, and Kafig (1961) reported successful storage of frozen Entamoeba histolytica for 91 days in 5% DMSO either slow-frozen to -35°C or frozen in dry ice before being placed in liquid nitrogen. Bemrick (1961) reported that best results were obtained using a 14.2% glycerol solution for freezing Giardia muris trophozoites. Fitzgerald and Levine (1961) froze Tritrichomonas foetus and Jeffery (1962) froze Plasmodium berghei and P. gallinaceum successfully in glycerol.

Collins and Jeffery (1963) reported success in freezing experimental malarias in DMSO and Hwang (1964) and Wang and Marquardt (1966) successfully froze Tetrahymena pyriformis in 10% DMSO.

Allain (1964) successfully froze eleven strains of Trypanosoma cruzi and T. dettoni and three species of Leishmania for 18 months in glycerol.

Meyer and Chadd (1967) reported 7% glycerol as the best concentration for freezing Giardia trophozoites when glycerol and DMSO were compared under identical slow-freezing conditions.

Lovelock (1953) using blood early proposed the mechanism of the protective action of glycerol against haemolysis during freezing and thawing. The theory states that glycerol functions by lowering the concentration of both the intracellular and extracellular salt in equilibrium with ice at any temperature below freezing, thereby preventing the salt concentration within the cell from rising to "a critically damaging level until the temperature is so low that the rate of damage is also low enough to be tolerable." This mechanism for the protective action of glycerol is still in general acceptance today. He (1954) also cites the following four characteristics of a good freezing preservative: low molecular weight, miscibility with salt solution, nontoxicity and ability to permeate the cell freely. Because DMSO has been reported to be toxic to certain organisms, glycerol appears to be the better protector during freezing.

MATERIALS AND METHODS

Serum Preparation

Blood was obtained from heavy white turkey hens (about 130 days old) by slitting the jugular vein and collecting the blood in Petri dishes. The dishes had been coated with physiological saline (0.7%) to prevent blood clots adhering to the sides. This technique yielded a higher percentage of serum and was easier to handle than that involving test tubes.

The dishes containing blood were covered and allowed to set undisturbed for two hours or were refrigerated (8.8°C) overnight before the serum was carefully removed using a Pasteur pipette. The serum was divided into groups, clear and cloudy. Hemolyzed serum was not used as Larson (1964) indicated that it was detrimental to multiplication of Histomonas.

When the sera were centrifuged, cloudy sera generally cleared. Sera collected at room temperature or refrigerated was centrifuged at 75 and 160 *g*, respectively, for 15 minutes at 0°C. The supernatant was pipetted off and again divided as to clear and cloudy. Continued refrigeration was found to yield more serum but of lower quality because of increased hemolysis. All tubes were dated and labeled as to quality. Sera were handled carefully to prevent undue contamination prior to autoclaving in the DeVolt's medium.

Sera were preserved in two ways. Some were quick-frozen at -78°C (dry ice and diethyl ether) while other sera were placed in a freezer at -16°C. All sera were stored at -16°C until added to DeVolt's medium when they were thawed and centrifuged to remove precipitated proteins. Clear sera produced less variable growth than did cloudy sera (Ruff, 1968).

Medium

A modified DeVolt's alkaline serum medium (DeVolt, 1943) was used for all cultures. DeVolt's medium consists of the following: 1000 ml distilled water, 9.0 g NaCl, 0.2 g CaCl, 0.4 g KCl, 0.2 g $NaHCO_3$, glucose, 20 ml turkey serum, and 20 ml of 0.2N NaOH to adjust to pH 9-10.

Modifications included the use of double glass distilled deionized water, 10 ml of turkey serum and no glucose in order to reduce growth of associated cecal bacteria. The pH was adjusted to 9.0-9.25 using 0.2N NaOH and a pH meter.[1]

A Cornwall automatic pipetter[2] was used to place 5 ml of the medium in each 16 x 75 mm Kimax screw cap tube. The tubes were loosely capped and autoclaved at 120°C, 15 lb pressure for 20 minutes. After autoclaving the caps were tightened and the tubes refrigerated at 8.8°C until needed. Medium over three months old was discarded as Larson (1964) and Ruff (1968) reported variable results using such medium.

Charcoal

Lumps of animal charcoal[3] used in the medium had an average weight of .155 $\pm$ .033 g/lump (Table XII). The term "lump" is used to characterize the irregular shape of the charcoal. Selecting uniform sizes of lumps can be done as demonstrated by results shown in Table XII of the appendix. This lump charcoal yielded less variable multiplication in the tubes and made counts easier than the finely ground charcoal previously used by Larson (1964) and Ostlind

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[1] Leeds and Northrup, Phila., Penn.

[2] Becton, Dickinson and Co., Rutherford, N. J.

[3] Fisher Scientific Co., Fairlawn, N. J.

(1966). It was placed in 150 x 16 mm Kimax screw-cap tubes and sterilized in dry heat at 160°C for 2 hours. The charcoal was stored at room temperature until needed. Unless indicated otherwise, a normal amount of charcoal in a culture tube was from 3 lumps (.418 ± .032 g) to 6 lumps (.803 ± .040 g) or enough to just fill the bottom curvature of the test tube.

Starch

Bacto rice powder[1], finely ground with a mortar and pestle, was placed in 60 x 17 mm homeopathic vials. The opening of one group of vials was only half covered with aluminum foil to facilitate starch removal with a scoop whereas the openings of the other vials were completely occluded. The foil in the latter group was punctured so that the starch could be shaken into those tubes requiring a less accurate record of multiplication of organisms. Both groups of vials were then capped with an additional sheet of foil which could be removed when the starch was used. Vials were sterilized in a dry heat oven at 150°C for 2 hours, care being taken not to char the starch.

Charcoal and 3.5-7.0 mg starch were added to warmed tubes of media just before the organisms were inoculated. The contents of the tubes were mixed gently by inverting, the organisms were added and the tubes incubated at 39.5°C in a water bath.

Source of Culture

Histomonas meleagridis were obtained from stock cultures which had been transferred six times for a total of 41 days in vitro culture. The original organisms were isolated 9-12 days after exposing turkeys to about 2,000 Heterakis gallinarum infective eggs.

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[1]Difco Laboratories, Detroit, Michigan.

Inoculation and Incubation

When starting an experiment, the number of organisms in a stock culture was determined by taking a count from the thoroughly mixed tube contents by means of a hemacytometer. The volume of culture necessary to transfer a desired number of organisms was calculated from this count. The tube was then mixed again and the determined volume extracted and inoculated into a warmed tube of DeVolt's medium. This procedure was continued until all the tubes had been inoculated. Aseptic technique was essential for optimum growth of Histomonas.

The tubes were then placed in a circulating water bath equipped with a mercury thermoregulator[1] and incubated at 39.5°C. Experimental groups consisted of 5 or 6 tubes per group.

Glassware

All glassware, except the Petri dishes, was acid cleaned. Pasteur pipettes used for removal of culture samples and graduated pipettes were placed in an acid bath (H_2SO_4 - $K_2Cr_2O_7$). After soaking for 24 hours, they were transferred to an upright automatic pipette washer[2] and flushed with tap water for at least 16 hours before they were removed and dried. The Pasteur pipettes were placed in large test tubes which then were plugged with cotton and capped with foil; the other pipettes were placed in metal containers. All pipettes were dry heat sterilized for 2-3 hours at 170°C.

All screw-cap tubes were placed in an acid bath of concentrated HNO_3 for a minimum of 24 hours. They were rinsed 9 times in tap, twice with distilled, and twice with double glass distilled deionized water, placed upside

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[1] E. H. Sargent and Co., Chicago, Illinois.

[2] Wm. Boekel and Co., Inc., Phila., Penn.

down in racks and allowed to dry. The tubes were sterilized along with the medium when needed. Caps for these tubes, except for the acid procedure, were cleaned in the same manner as the tubes.

Counting

A Levy ultra plane hemacytometer with improved Neubauer ruling was used for counting the cultures of Histomonas. It consists of a thick glass slide with two central platforms, each engraved with rulings and covering an area of 9 square millimeters. The glass cover slip is suspended 0.1 mm above the central platform creating two identical chambers. The number of organisms per milliliter is calculated according to the following formula:

$$\frac{N}{9}\ (10)\ (1000) = \frac{N\ 10^4}{9} = \text{organisms/cc}$$

Pasteur pipettes (225 x 6 mm) were used for transfers of the organism to the hemacytometer. The pipette was filled with a thoroughly mixed suspension, the tip was touched to the edge of the cover slip, and enough liquid was allowed to flow under it to just fill the chambers.

The criterion used in counting only live organisms were refractivity, presence of starch granules and/or motility. Periodically, especially in old cultures, organisms appeared which were referred to as "ghosts" and were thought to be moribund or dead organisms. They were much less refractile, slightly bluish with the outer limiting membrane appearing only faintly, and were not included in the growth counts. In addition, where zero or very low count tubes were encountered on the first and succeeding counts, these tube counts were not included in the average count for that group. Therefore, some groups contained fewer than 6 tubes.

Wherever the terms growth or multiplication are used in this thesis they refer to the number of Histomonas per milliliter or cubic centimeter of

medium.

Transfer of Organisms

Transfers to fresh medium were made every two weeks by removing a sample from the bottom of an undisturbed stock culture tube with a sterile Pasteur pipette and placing 2-3 drops into a new tube of complete medium. Population counts were not normally taken before transfer but rather samples from the bottom of the culture tubes were placed on a glass slide and examined for abundance of histomonads. A good number of organisms for transferring was about 3,000-5,000 Histomonas per hi-dry (450x) microscope field.

At least four tubes were maintained as stock cultures while two cultures from previous transfers were kept in reserve. Some tubes from each transfer were kept in two separate water baths (39.5°C) and incubator (40.0°C) to insure the survival of the experimental strain in the event of power failure or malfunction of one of the units.

Statistical Analysis

All appropriate original data in these experiments was subjected to an analysis of variance test to determine the degree of significance between the comparative multiplication of the organisms in each group. A probability distribution known as the F distribution where $p < .05$ was accepted as significant, i.e. the probability that the differences between groups shown by the data were actually due to treatments or variables rather than chance differences in sampling.

The original data from Experiment 1 dealing with agitation of the organism was converted to counter logs to facilitate a more meaningful statistic; all other data was used in its original form.

Appendix

All tables usually duplicating data in graphs are in the appendix. Only one table, Table XI, occurs in the text. In addition, the appendix contains certain supplemental data which is summarized in the text.

RESULTS AND DISCUSSION

Effects of Agitation on Multiplication

The first factor investigated relative to the culturing of histomonads was the effect of agitation of the culture tubes on multiplication of histomonads within the tubes. Each tube in experiment 1, trials 1 and 2, received an inoculum of 9,000 and 3,770 organisms, respectively. Determinations of growth response were initiated 48 hours postinoculation. There were seven and eight groups (five tubes each) in trials 1 and 2, respectively. Because of technique problems and an unexplained variability in multiplication rates of the organism, some tubes had to be discarded in trial 1 as no growth was evident on the first and subsequent counts; thus the average counts of groups 3, 6, and 7 were based on three tubes.

The following schedule of agitation of culture tubes was followed for trials 1 and 2: groups 1 were agitated and population counts taken daily, and all other groups were agitated and counted twice; groups 2 on day 3 and 5; groups 3 on day 4 and 6; groups 4 on day 5 and 7; groups 5 on day 6 and 8; groups 6 on day 7 and 9 (trial 2 only); groups 7 on day 8; group 8 on day 9 (trial 2 only). This method of making population counts revealed three separate relationships; 1) daily counts and agitations of the same cultures, 2) first counts of all cultures, one previously undisturbed group being counted and agitated each day and 3) second counts of cultures (groups 2-5, trial 1, and groups 2-6, trial 2), each made 48 hours after the initial agitation and count.

When counts were made of organisms, each tube was removed in turn from the water bath and gently inverted three times before quickly removing a

sample with a Pasteur pipette and filling the hemacytometer chambers.

The results of these studies are shown in Fig. 1 and given in Tables I and II. It was not possible to analyse the data from trial 1 because of the variability in the number of tubes per group. However, no significant difference in population levels was observed in trial 2 between the three methods of agitation which indicates that daily agitation has no apparent adverse effect on multiplication of the organism.

Effects of Starch on Multiplication

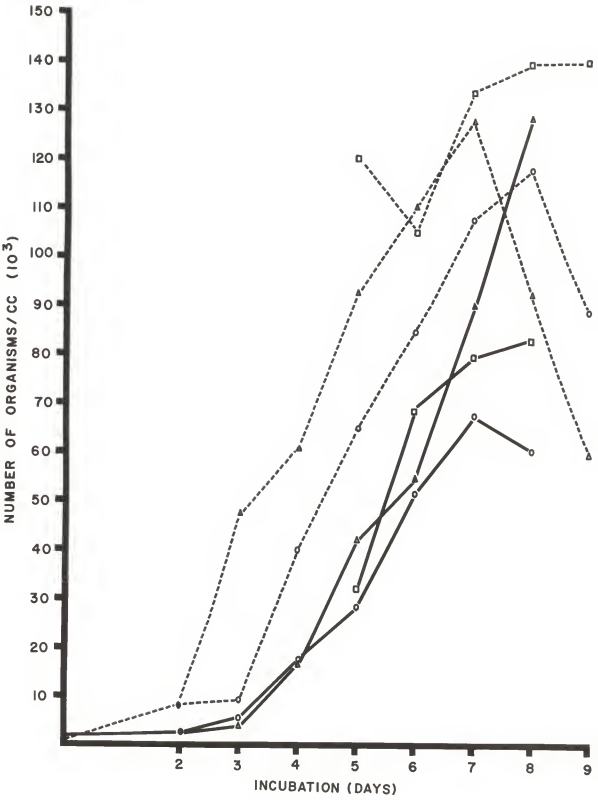
The purpose of this experiment was to determine the optimum amount of starch for continuous and consistent multiplication of the organism *in vitro*. Three groups of six tubes each received an inoculum of 265,830 organisms per tube. The amount of starch used in each group was 3.5 $\pm$ 0.0194 mg, 7.0 $\pm$ 0.0388 mg and 14.0 $\pm$ 0.0776 mg, respectively. Population counts were made after 48 hours of undisturbed incubation and thereafter every other day for eight days with a final count on the 15th day postinoculation.

The effects of different quantities of starch on multiplication of the organism are given in Table III, Fig. 2. The cultures receiving 3.5 mg of starch multiplied at a significantly slower rate than did the cultures receiving 7.0 mg or 14.0 mg of starch. The cultures receiving 7.0 mg of starch multiplied most rapidly except on day 15 postinoculation when this culture group was surpassed by the cultures receiving 14 mg. There was, however, no significant difference between the overall comparative multiplication of these two groups receiving the larger amounts of starch.

Effects of Charcoal on Multiplication

The exact function of charcoal in the tubes is not completely understood. It has been suggested that it absorbs harmful products of metabolism

Fig. 1. Effects of agitation of culture tube on multiplication of Histomonas meleagridis, Experiment 1, trials 1 and 2. Legend: (o) daily counts and agitations of the same cultures, (Δ) first counts of all cultures, one previously undisturbed group being counted and agitated each day, (□) second counts of cultures, each made 48 hours after the initial agitation and count. Solid line delineates trial 1, and broken line delineates trial 2.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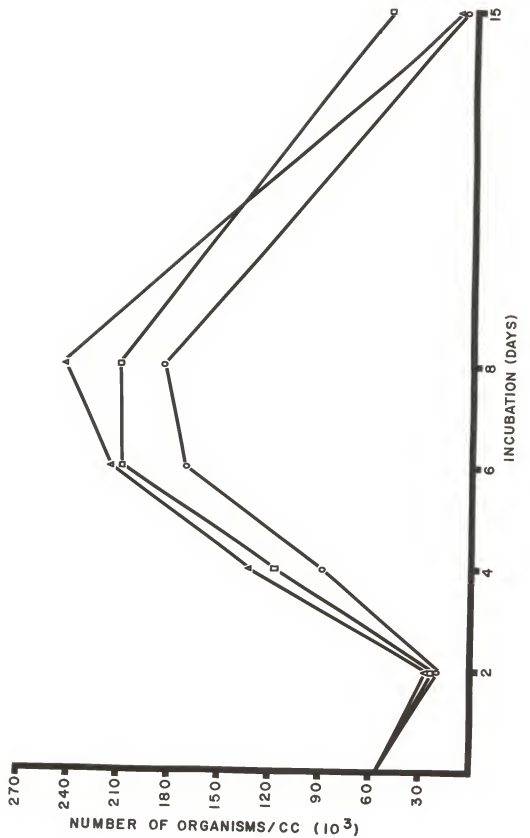
INCUBATION (DAYS)

Fig. 2. Effects of amount of starch on multiplication of Histomonas meleagridis, Experiment 2. Legend: (o) 3.5 ± 0.0194 mg, (Δ) 7.0 ± 0.0388 mg and (□) 14.0 ± 0.0776 mg of starch/tube of culture.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INCUBATION (DAYS)

because tubes with charcoal support growth longer than those without charcoal. This experiment was designed to determine the optimum amount of charcoal for rapid multiplication and sustained survival of the organism.

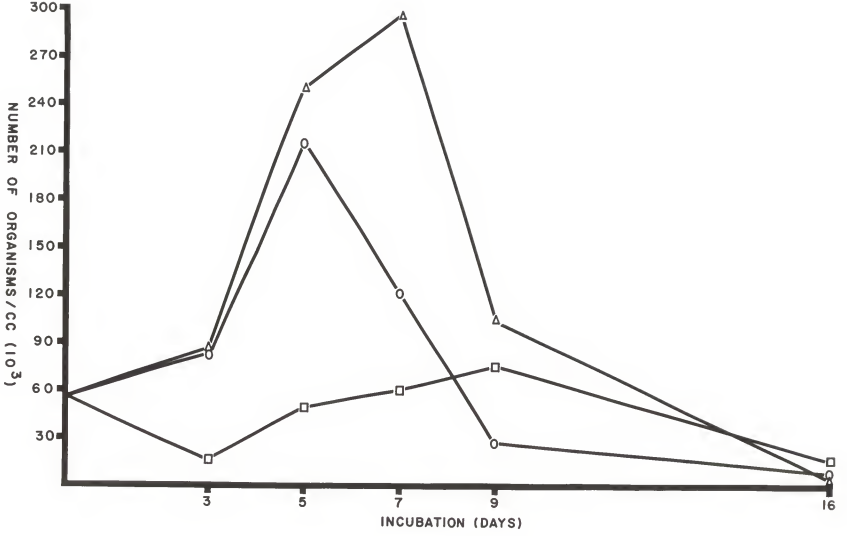
Three groups of six tubes each received an inoculum of 265,830 organisms/tube. The amount of charcoal for each group was one lump (.155 $\pm$ .033 g), three lumps (.418 $\pm$ .032 g) or 6 lumps (.803 $\pm$ .040 g), respectively. The selection of lumps was made on a visual basis, care being taken to choose lumps of nearly uniform size (Table XII). Counts were initiated after 72 hours of incubation and thereafter were made every other day to day 9. The final count was made on day 16 postinoculation.

The effects of lump charcoal on multiplication are shown in Fig. 3 and Table IV. The groups with one, three and six lumps of charcoal reached peak counts of multiplication on days 5, 7 and 9, respectively. The groups receiving .155 $\pm$ .035 g and .418 $\pm$ .034 g of charcoal multiplied as well as declined more rapidly than did the group receiving .803 $\pm$ .042 g of charcoal. Even though the group receiving the greatest amount of charcoal produced a significantly inferior population growth the population was more stable over the test period. This suggests that an overabundance of charcoal might inhibit high multiplication rates but also tends to better sustain a culture over a longer time.

Effects of Age of Serum, Method of Preservation and Use of Plasma on Multiplication

The purpose of this experiment was to determine what effect the age of serum used and method of preservation have on multiplication of Histomonas in DeVolt's medium. Also incorporated in this experiment was one group using turkey plasma instead of turkey serum in the medium in order to observe its effects on the multiplication of the organism.

Fig. 3. Effects of amount of lump charcoal on multiplication of Histomonas meleagridis, Experiment 3. Legend: (o) .155 ± .035 g, (Δ) .418 ± .034 g and (□) .803 ± .042 g of lump charcoal.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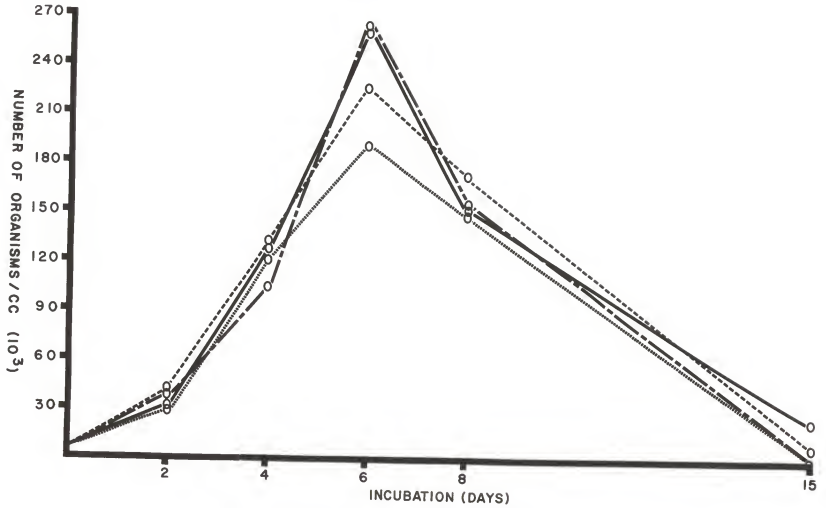
INCUBATION (DAYS)

Eight groups of 6 tubes each were used in this experiment. Each tube received an inoculum of 23,300 organisms. Counts were begun after 48 hours of undisturbed incubation and continued every other day for 8 days with a final count on day 15 postinoculation.

The effects of age, method of serum preservation and the use of plasma on multiplication are shown in Figs. 4 and 5 and Table V. There was no significant difference in multiplication between the 5 groups slow-frozen for varying lengths of time which indicated that the age of serum used does not affect multiplication. Likewise, in reference to the method of preservation by freezing, there was no significant difference between the multiplication levels of the serum groups slow- or quick-frozen. Even though both the plasma and quick-frozen serum groups appeared to have higher levels of multiplication than did the slow-frozen serum groups, these levels were not high enough to be of significance. The refrigerated serum group, however, showed a highly significant difference in multiplication at its growth peak which was followed by a sharp drop to a level similar to the other groups.

These data indicated that serum quick-frozen at $-78^{\circ}C$ and stored at $-16^{\circ}C$ and serum slow-frozen and stored at $-16^{\circ}C$ for varying periods of time were not significantly different in their effects on multiplication. Plasma produced growth similar to serum and could possibly be substituted for serum in the medium. Refrigerated serum produced significantly greater levels of multiplication but only for a short time. This trend suggested that possibly protein denaturation in the frozen groups caused the poorer growth as the best growth occurred in the refrigerated serum which would have had the least degree of protein denaturation.

Fig. 4. Effects of different aged slow-frozen sera on multiplication of Histomonas meleagridis, Experiment 4. Legend (time frozen): - - - -, 780 days; —— - - ——, 510 days; ·····, 420 days; ————, 6 days (control).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NUMBER OF ORGANISMS / CC (10³)
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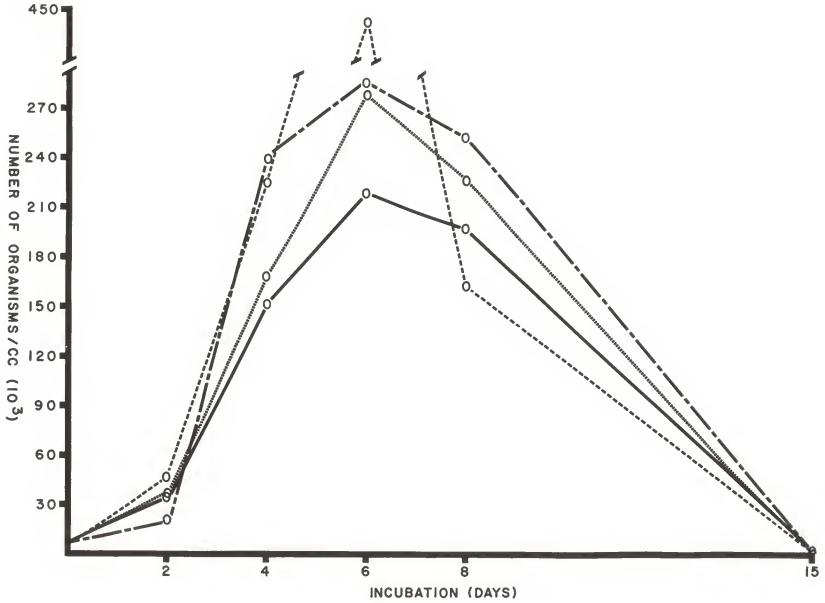
INCUBATION (DAYS)

Fig. 5. Effects of refrigerated, slow and quick frozen sera on multiplication of Histomonas meleagridis, Experiment 4. Legend (time): ·····, 6 days (slow-frozen, plasma); – – – –, 450 days (refrigerated, serum); —·—·—, 6 days (quick-frozen, serum); ———, 6 days (slow-frozen, serum) (Control).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INCUBATION (DAYS)

Multiplication Rate

This experiment was designed to determine the length of time a culture could be maintained before cultures had to be transferred. Knowledge of the typical growth curve of Histomonas under prescribed conditions is important for maintaining stock cultures and determining the best time to transfer cultures.

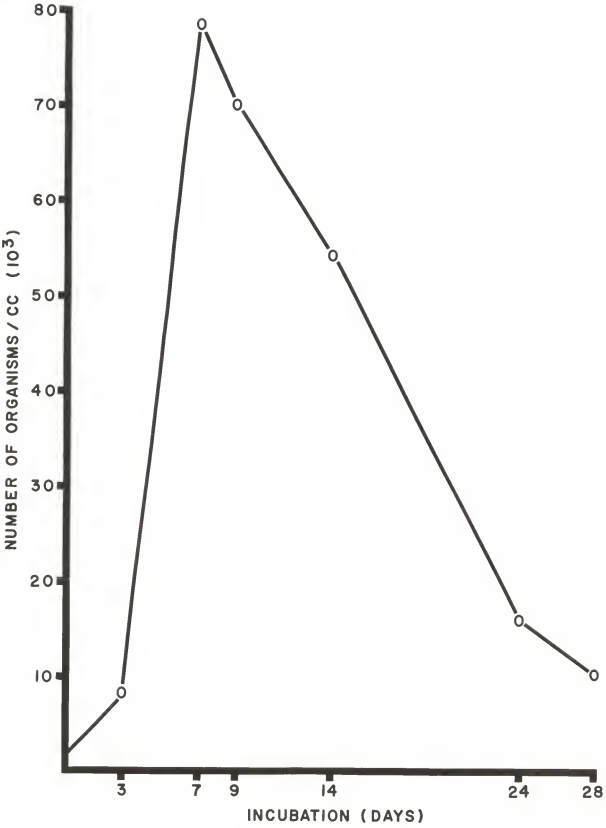
Seven tubes received an inoculum of 11,220 organisms/tube, 7.0 mg of starch, and each tube received enough charcoal to fill the bottom curvature. Average counts were determined on the days shown in Fig. 6 and Table VI. The results indicated a rapid rate of multiplication which peaked on the seventh day postinoculation and then began to decline. Organisms were still observed in transferrable numbers up to 28 days postinoculation. It can be concluded that 7-day-old cultures are best for making transfers to fresh medium. However, one could resort to older cultures for making transfers if necessary.

Effect of Age of Culture on Rate of Multiplication

The comparative rates of multiplication of one-, two-, and three-week-old cultures were used to determine the optimum age of a culture for experimental inoculations.

The three stock cultures were established as follows; one tube was inoculated with organisms and allowed to grow one week at which time a subculture was made from this tube and both tubes were incubated for one week. A sample from the second tube was then subcultured to a third tube and all three tubes incubated for an additional week. At this time the three tubes contained three stock cultures all from the same initial source and each differing in age by one week.

Fig. 6. Multiplication and survival of Histomonas meleagridis, Experiment 5.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NUMBER OF ORGANISMS / CC (10³)
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INCUBATION (DAYS)

From these three tubes, three groups of six tubes each received an inoculum of 11,220 organisms from its respective stock culture tube. Population counts were begun two days postinoculation and made every other day for 12 days.

The effects of age of culture are shown on Fig. 7 and Table VII. The results indicated that there was no significant difference between the comparative multiplication of the different aged cultures. The late growth peak and sustained growth of the tubes from the three-week-old culture along with its lowest growth number of the three groups indicated that younger cultures probably are the best stock cultures from which to subculture. All tubes, however, attained excellent growth peaks which indicated that older cultures could still be used as stock cultures.

Anaerobiasis

The effects of anaerobiasis (nitrogen) on multiplication was studied in this experiment.

Each of 12 tubes was inoculated with 92,120 organisms. The air was replaced in 6 tubes by gently blowing nitrogen gas into the space over the culture fluid. The other 6 tubes received no nitrogen treatment and served as controls. Population counts of the organisms were made on four consecutive days starting on day 3 postinoculation.

Results of this experiment are shown in Fig. 8 and Table VIII. The multiplication in the tubes containing nitrogen gas was significantly and consistently lower than the ones containing oxygen. In addition, there was a more pronounced lag phase in multiplication in the nitrogen cultures than in the controls. These results indicated that nitrogen gas was unsuitable for achieving anaerobic conditions.

Fig. 7. Comparative multiplication rates of Histomonas meleagridis in cultures inoculated with different ages of stock cultures, Experiment 6. Legend: ————, 1 week; —— - ——, 2 weeks; - - - - - - - , 3 weeks.

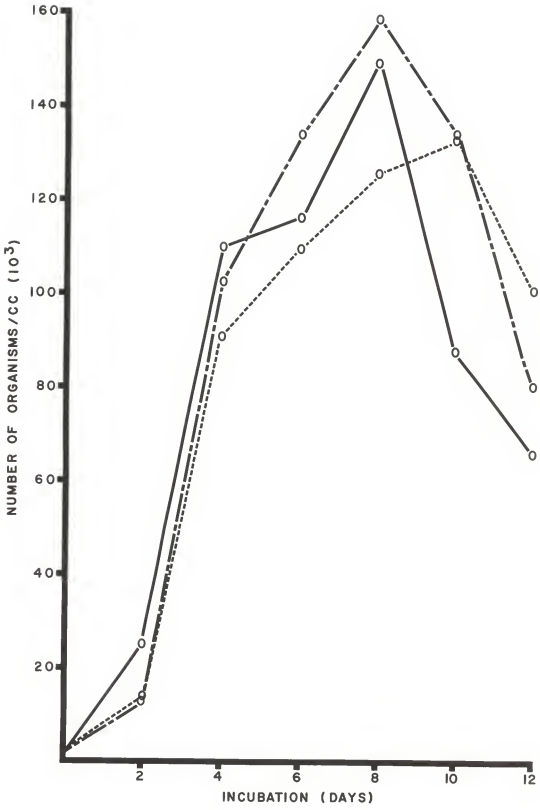
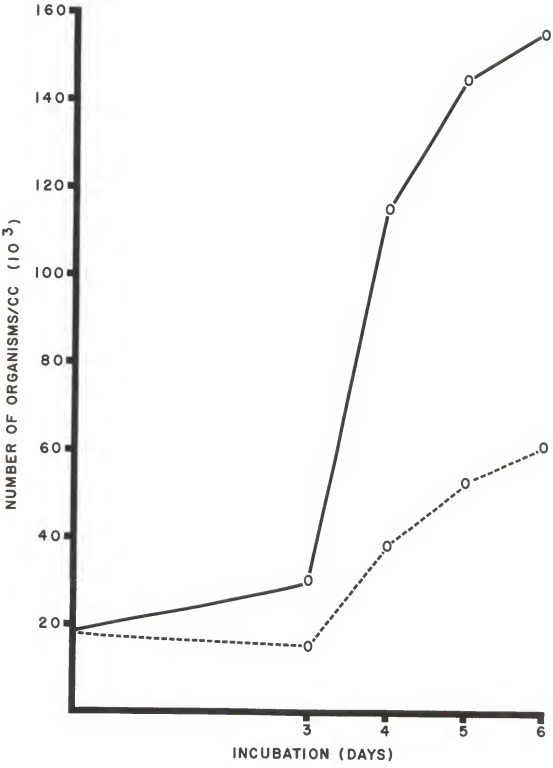

160
140
120
100
80
60
40
20
NUMBER OF ORGANISMS/CC (10^3)
2
4
6
8
10
12
INCUBATION (DAYS)

Fig. 8. Effect of anaerobiasis on multiplication of Histomonas meleagridis. Experiment 7. Legend: ———, control; — — —, anaerobic.


160
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NUMBER OF ORGANISMS/CC (10 3)
3
4
5
6
INCUBATION (DAYS)

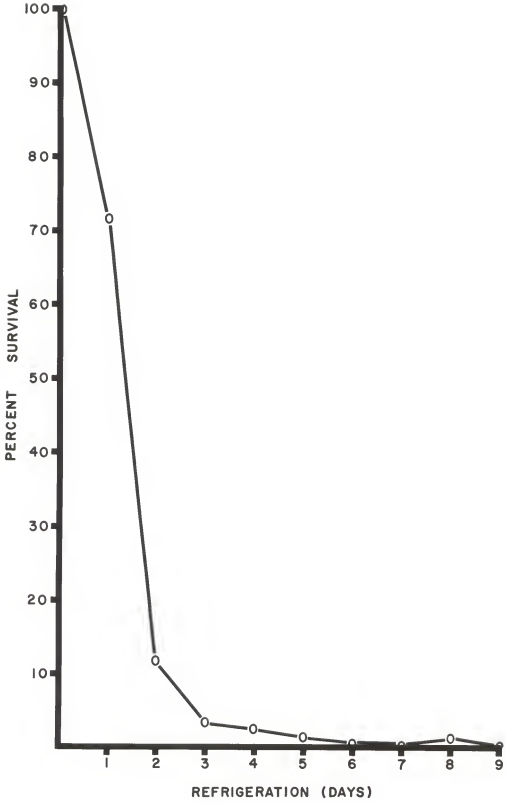
Effect of Refrigeration on Multiplication

In a pilot experiment several tubes with good growth of histomonads were refrigerated and examined daily to detect any decline in numbers. Because some of these organisms were able to survive refrigeration (8.8°C) for three days, a second experiment was done to determine per cent survival. In addition, some tubes were frozen and the favorable results from this trial prompted later experiments using glycerol as a preservative.

To determine per cent survival following refrigeration, each of 30 tubes received an inoculum of 11,220 organisms and all were incubated for 7 days. Population counts were made in each tube, 27 of which had sufficient numbers of organisms for use in this refrigeration experiment. These cultures were divided on the basis of numbers of organisms into nine groups of three tubes each; the lower count groups were removed from the refrigerator and counted before those with higher original counts. The results were recorded in terms of per cent survival per group. After counting, subcultures were made from each tube for that day and combined into one tube, incubated and checked for survival nine days later to determine the organism's ability to recover from thermal shock.

The per cent survival of the organisms are shown on Fig. 9 and Table IX. The greatest per cent casualty occurred within 24-48 hours of exposure when the per cent survival declined from 72.1% to 11.9%. Organisms continued to live in the tubes removed until the ninth day when no living organisms were observed. In addition, all but the last subculture taken from the original refrigerated tubes showed some degree of recovery with the quickest and best recovery in the tubes exposed to refrigeration for the shortest time.

Fig. 9. Survival of Histomonas meleagridis in a normal culture medium after refrigeration (8.8°C), Experiment 8.


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
1
2
3
4
5
6
7
8
9
REFRIGERATION (DAYS)

Freezing

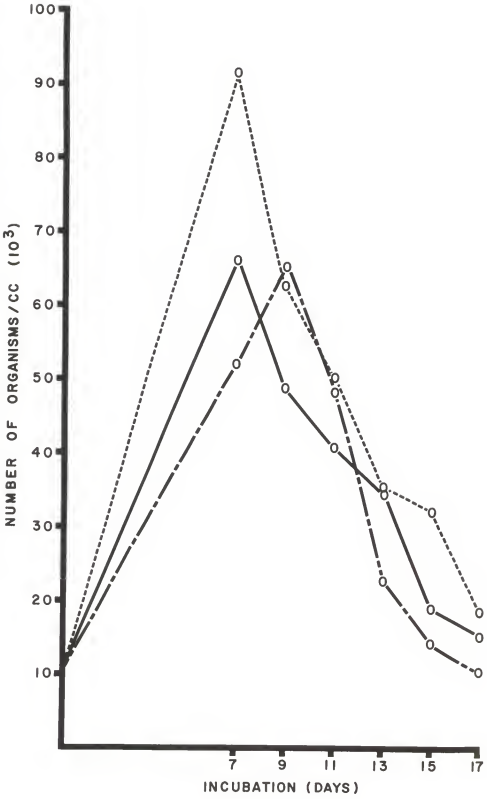
Survival of the organism after freezing both with or without preservative was investigated in this experiment. As previously stated, some tubes of high population count were frozen as part of the refrigeration experiment. Eight tubes of organisms were quick-frozen (dry ice in diethyl ether at $-78^{\circ}C$) and 8 were slow-frozen (cold storage freezer at $-16^{\circ}C$). Those tubes that had been quick-frozen were stored with the other 8 at $-16^{\circ}C$. Periodically tubes were removed, thawed rapidly in a water bath ($39.5^{\circ}C$) or slowly at room temperature and examined using the criteria for living organisms previously described. Viability was ascertained by subculturing in a new tube of DeVolt's medium.

In one tube that had been quick-frozen, stored at $-16^{\circ}C$ for 15 days and rapidly thawed, "ghosts" or faint outlines of Histomonas were observed. Three days after subsequent subculturing definite signs of life were again observed. When examined a week later, organisms were still present but in fewer numbers.

The effect of glycerol, a freezing preservative, on the growth cycle of the organism and on survival of the organism upon freezing was examined. However, before glycerol could be used as a preservative in the frozen tubes, it was necessary to determine its effect on the organisms under normal growth conditions. Each of 18 tubes received an inoculum of 52,000 organisms and were then divided into three equal groups, two of which received 5% and 10% glycerol, respectively, while the third group served as a control. Counts were initiated on day seven postinoculation and thereafter made on alternate days to day 17.

The results as shown in Fig. 10 and Table X indicated no significant difference between the two glycerol groups and the control. The 5% glycerol group produced the best general growth throughout the experiment but the level

Fig. 10. Effect of glycerol on multiplication of Histomonas meleagridis under normal growth conditions, Experiment 9. Legend: ———— , control; – – – – –, 5% glycerol; —— - ——, 10% glycerol.


100
90
80
70
60
50
40
30
20
10
NUMBER OF ORGANISMS/CC (10^3)
7
9
11
13
15
17
INCUBATION (DAYS)

was not significant. Glycerol therefore had no apparent adverse effect on the multiplication of the organism under normal growth conditions and may actually tend to stimulate growth at certain concentrations.

The final experiment dealing with the preservation effects of glycerol on the organisms upon freezing was used as a pilot experiment for further research. Several tubes from the preceding experiment containing the 5-10% glycerol and exhibiting the highest population counts were frozen and later thawed, subcultured and examined for viability.

The results from freezing Histomonas with the preservative glycerol are given on Table XI. When examined after a period of incubation, living organisms were observed in greater abundance than was observed previously in the tubes frozen without glycerol with the best survival in those tubes which had been quick-frozen and slow-thawed at room temperature. This indicated that glycerol probably had a protective action on the organism during freezing.

TABLE XI

Survival of *Histomonas meleagridis* after freezing and thawing in the presence of 5–10% glycerol.[a] Experiment 9.

Frozen (Days)	Quick-Freeze		Slow-Freeze	
	Quick-Thaw	Slow-Thaw	Quick-Thaw	Slow-Thaw
5	−			±
8	−			−
15	−	±	±	−
18		±	±	
22		+	±	
26		+	−	
33		+	−	

[a] ± = "ghosts"; + = survival; − = death.

SUMMARY

Daily agitation and counting of Histomonas meleagridis cultures in a modified DeVolt's medium and the age of culture used for inoculations were found to have no adverse effects on rate of multiplication of the organism. The age of serum used in the medium did not affect the rate or degree of multiplication; however, the method of preservation of the serum did affect multiplication in that refrigerated serum stimulated more rapid short term multiplication than did frozen serum. There was no difference between multiplication of histomonads in media containing plasma or serum.

The amount of starch and charcoal used in the medium affected the multiplication of the organism. A significantly lower growth level was observed in 5 cc of medium containing 3.5 mg of starch (finely ground Pacto rice powder) than in medium containing 7.0 mg or 14.0 mg of starch. When .803 g of animal charcoal was used in the medium, multiplication was less than that in cultures receiving .155 g or .418 g of charcoal.

The peak in multiplication of the organism occurred at day 7 postinoculation and the organisms survived for 28 days in vitro. Nitrogen gas was found unsuitable for maintaining anaerobic conditions for growth.

Thermal shock by refrigeration (8.8°C) was very detrimental to Histomonas with the greatest mortality occurring in the first 48 hours. Glycerol, a freezing preservative, in 5% and 10% concentrations had no apparent adverse effect on the organism under normal growth conditions and there were indications it may function as a growth stimulant in certain concentrations. The organisms survived freezing and thawing with and without glycerol as dem-

onstrated by successful subculturing. However, quick-freeze using dry ice in diethyl ether at -78°C and slow-thaw (room temperature) gave the most uniform results.

APPENDIX

TABLE I

Influence of Agitation of Cultures on the Multiplication of Histomonas meleagridis. Experiment 1, Trial 1.

Postinoculation and Agitation (Days)	Average Number/cc						
	Groups						
	1	2	3	4	5	6	7
2	2,960						
3	5,380	3,580					
4	17,890		16,700				
5	28,312	32,350		42,030			
6	51,700		69,030		54,700		
7	67,350			79,530		89,560	
8	60,170				83,050		128,200

TABLE II

Influence of Agitation of Cultures on the Multiplication of Histomonas meleagridis. Experiment 1, Trial 2.

Postinoculation and Agitation (Days)	Average Number/cc							
	Groups							
	1	2	3	4	5	6	7	8
2	8,500							
3	9,300	47,400						
4	39,900		60,700					
5	64,840	120,300		92,300				
6	84,620		105,400		110,160			
7	107,500			133,500		127,700		
8	117,700				139,500		92,010	
9	88,500					139,860		59,860

TABLE III

Effects of starch on the multiplication of Histomonas meleagridis. Experiment 2.

Postinoculation (Days)	Average Number/cc		
	Starch (mg)		
	3.5	7.0	10.5
2	20,436	24,010	23,283
4	89,480	133,880	117,850
6	170,880	217,800	209,610
8	184,110	243,800	211,760
15	6,360	10,980	52,260

TABLE IV

Effects of lump charcoal on multiplication of *Histomonas meleagridis*. Experiment 3.

Postinoculation (Days)	Average Number/cc		
	Charcoal (g)		
	.155	.418	.803
3	84,040	86,800	17,360
5	215,500	250,260	49,560
7	122,730	295,600	62,180
9	28,100	105,240	76,600
16	991	641	16,610

TABLE V

Comparative multiplication of Histomonas meleagridis in differently aged, frozen and refrigerated turkey sera and plasma.
Experiment 4.

Group	Sub-group	Serum		Average Number/cc				
		Treatment	Storage (Days)	Postinoculation (Days)				
				2	4	6	8	15
I	A	SF	780	43,110	133,810	227,450	173,280	9,380
	B	SF	510	40,330	114,680	264,310	157,160	1,620
	C	SF	420	29,110	122,360	190,130	149,410	1,890
	D	SF	6	30,060	129,750	261,630	152,550	25,610
II	E	SF[a]	6	37,530	170,910	280,680	228,710	275
	F	R	450	48,010	227,730	444,600	164,600	1,000
	G	QF	6	22,810	240,380	287,560	254,480	460
	H	SF	6	37,730	154,060	221,060	199,060	460

[a] plasma
SF = Slow Frozen, -16° C
R = Refrigerated
QF = Quick Frozen, -78° C, stored at -16° C

TABLE VI

Optimum multiplication of Histomonas meleagridis in culture. Experiment 5.

Average Number/cc					
Days					
3	7	9	14	24	28
8,310	78,800	70,100	54,760	16,200	10,550

TABLE VII

Comparative multiplication rates of Histomonas meleagridis started from different aged stock cultures. Experiment 6.

Postinoculation (Days)	Average Number/cc		
	Age of Stock Culture (Weeks)		
	1	2	3
2	25,500	13,900	14,100
4	110,100	103,000	91,200
6	116,100	134,000	110,500
8	148,800	158,700	126,300
10	88,700	134,500	133,700
12	66,100	80,700	101,100

TABLE VIII

Comparative multiplication of Histomonas meleagridis cultures grown under aerobic and anaerobic conditions. Experiment 7.

Condition	Average Number/cc			
	Days			
	3	4	5	6
Aerobic	31,200	116,000	145,500	155,000
Anaerobic	15,500	38,300	53,300	61,800

TABLE IX

Survival of Histomonas meleagridis in a normal culture media upon exposure to refrigeration (8.8°C). Experiment 8.

Refrigeration (Days)	Average Number/cc		Survival (%)	Recovery[a]
	Before Refrigeration	After Refrigeration		
1	62,000	44,730	72.10	+
2	192,700	22,930	11.90	+
3	202,700	7,530	3.72	+
4	226,800	5,860	2.50	+
5	238,100	3,110	1.30	+
6	239,300	1,630	0.67	+
7	280,600	920	0.32	?
8	321,000	3,900	1.20	+
9	116,000	0	0.00	-

[a]Recovery indicates signs of life after subculturing and three days of incubation.

TABLE X

Effect of solutions of glycerol on multiplication of Histomonas meleagridis under normal growth conditions. Experiment 9.

Incubation (Days)	Average Number/cc		
	Glycerol (%)		
	5	10	Control
7	92,200	52,800	66,200
9	63,300	65,800	49,500
11	50,600	48,100	41,800
13	36,300	23,300	34,500
15	32,700	14,700	19,300
17	18,800	11,000	15,300

TABLE XII

Method for Determination of the Standard Deviation of the Weights of Lump Animal Charcoal. Experiment 3.

	Number of Lumps		
	1	2	3
Sample Weights (g)	.167	.393	.772
	.132	.395	.826
	.195	.439	.843
	.202	.419	.799
	.135	.394	.743
	.115	.425	.785
	.160	.399	.866
	.107	.494	.859
	.202	.382	.797
	.137	.446	.759
Total	1.552	4.186	8.049
Mean	.155	.418	.804
Standard Deviation	.033	.032	.040

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FACTORS AFFECTING THE IN VITRO CULTIVATION OF HISTOMONAS MELEAGRIDIS

by

RANDALL M. MCKEE

B.A., Concordia College, Moorhead, Minnesota, 1967

AN ABSTRACT OF A MASTER'S THESIS

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DIVISION OF BIOLOGY

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Manhattan, Kansas

1969

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The peak in multiplication of the organism occurred at day 7 postinoculation and the organisms survived for 28 days in vitro. Nitrogen gas was found unsuitable for maintaining anaerobic conditions for growth.

Thermal shock by refrigeration (8.8°C) was very detrimental to Histomonas with the greatest mortality occurring in the first 48 hours. Glycerol, a freezing preservative, in 5% and 10% concentrations had no apparent adverse effect on the organism under normal growth conditions and there were indications it may function as a growth stimulant in certain concentrations. The organism survived freezing and thawing with and without glycerol as demonstrated by successful subculturing. However, quick freeze using dry ice in diethyl ether at - 78°C and slow thaw (room temperature) gave the most uniform results.

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