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Some studies on the biology of a species of Hexamita derived from the oyster, Crassostrea virginica (Gmelin).

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SOME STUDIES ON THE BIOLOGY OF A SPECIES OF *HEXAMITA* DERIVED FROM THE OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN)

by

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ABSTRACT

The trophozoites of Hexamita inflata, a small anaerobic flagellated protozoan associated with oyster epizootics, have been isolated and cultivated in axenic cultures for the first time using a medium of low oxidation-reduction potential. Contaminating organisms were eliminated by the combined use of serial transfers and antibiotics. The isolation medium contained: sea water; peptone; glucose; yeast extract; cysteine; egg-yolk suspension or beef serum; and the antibiotics penicillin, streptomycin and mycostatin.

A brief morphological study of H. inflata in axenic culture was carried out. It confirmed that the species involved was H. inflata as previously claimed but inadequately described by others. Reproduction of H. inflata was by longitudinal binary fission only. No cysts were observed in cultures even under various conditions of temperature, pH, redox potential and salinity. However, the formation of spherical cells of Hexamita, with or without the full complement of flagella, was noted under some unfavourable conditions.

The effects of physical factors on growth were also studied. The flagellate was capable of growing over a wide range of temperature (5 C to 25 C), of hydrogen-ion concentration (pH 4.5 to 8.5), and of salinity (3 to 28‰); and required a reducing or anaerobic environment. These conditions were similar to those in the natural habitat of

the organism.

In nutritional studies, it was demonstrated that the function of the egg-yolk suspension was to supply an unsaturated fatty acid --- a requirement readily satisfied by linoleic acid and to some extent, oleic or linolenic acid. Attempts to replace the peptone by mixtures of amino acids were not successful; but a partially defined medium containing glucose, cysteine, vitamins, peptone and salts was formulated.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The species of Hexamita with which this study was concerned is a small (8x16 μ) flagellated anaerobic protozoan, the trophozoites of which occur most abundantly in the visceral mass of dead oysters (gapers) in the winter. It or a closely similar flagellate has at times been implicated in various European and American oyster epizootics (Mackin, Korringa, and Hopkins, 1952; Stein, Denison, and Mackin, 1961; Laird, 1961; Scheltema, 1962) and a condition of Canadian oysters known as Malpeque disease (Richardson, 1939; Drinnan, Henderson, and Khouw, 1961; Laird, 1961). Certes (1882) and Laird (1961) identified the species commonly found in oysters as H. inflata but others (Mackin et al., 1952; Stein et al., 1961; Marteil et al., 1962; Scheltema, 1962) have hesitated to assign a species designation. A definitive demonstration of the etiological role of Hexamita, however, has not yet been accomplished.

Our work on this organism as well as that of others has been from the beginning handicapped because of the lack of suitable techniques for its cultivation and study. It was in order to correct this deficiency that the studies reported in this thesis were initially undertaken. During the course of this work, a method for the isolation and cultivation of the trophozoites in axenic culture was

developed, which then permitted some morphological and nutritional studies; and subsequently the development of a partially defined synthetic medium.

Brief Resume of the Genus Hexamita.

The genus Hexamita was erected by Dujardin in 1841 to include three species of diplozoic flagellates possessing six flagella. Actually the organism has since been found to have eight flagella, six situated anteriorly, and two, posteriorly; thus it was more descriptively called Octomitus. Nevertheless the misnomer has priority and may thus stand (Mackinnon and Hawes, 1961). The genus is represented by a wide array of free-living, symbiotic, commensalic, parasitic and even hyperparasitic species. In addition to a free-living form, H. nodulosa, occurring in stagnant water, species of Hexamita have also been recorded in nematodes, trematodes, leeches, insects, fishes, amphibians, reptiles, birds, rodents and monkeys (McNeil, Hinshaw, and Kofoid, 1941). They have not, however, been recorded in man. H. inflata has also been recorded from oviparous oysters, for example, Ostrea edulis (Certes, 1882), and viviparous oysters, such as Crassostrea virginica (Laird, 1961). Other records exist also of the occurrence of unidentified Hexamita species in O. lurida (Stein et al., 1961), Cr. angulata (Marteil et al., 1962) and Cr. gigas (Drinnan and Khouw, 1962, unpublished observations).

The morphological characteristics of Hexamita have been described by various authors including Kudo (1946),

Grasse (1953), Hall (1953) and Mackinnon and Hawes (1961). The trophozoite is characterized by a bilaterally symmetrical pyriform body which bears six anterior and two trailing posterior flagella and which has two axostyles running along its length. The endoplasm is vacuolated and contains numerous refractile granules. There are two nuclei situated anteriorly. In spite of the fact that species of Hexamita show great variations and considerable overlapping in body size, ranging from 6-12 μ by 2-5 μ in H. meleagridis to 17-20 μ by 9-15 μ in H. inflata, size and shape differences are still of importance in species identification. Encystment has been observed in some species. The binucleate cysts have been described as being small, oval and seldom more than half the size of the corresponding trophozoites. Slavin and Wilson (1960) have reported the occurrence of two types of cysts in H. meleagridis. They were observed in mucous clots of the bowel scrapings of infected turkeys. One was spherical and about half the size of the trophozoite, with a double nucleus situated at one end. Flagella were usually retained in this type of cyst. The second type was not flagellated and was termed a resistant cyst because of its greater resistance to drying. It was described as being symmetrically oval with a nucleus situated in each of the blunt, rounded ends.

Reproduction of the trophozoites is usually by simple longitudinal binary fission, but reproduction has also been said to involve multiple fission and schizogony (Moore, 1923;

Davis, 1925; Slavin and Wilson, 1960). In the more recent report by Slavin and Wilson (1960) on H. meleagridis, it was claimed that schizonts incubated in mucous clots developed into merozoites which in turn were converted into tail-less, aflagellate motile forms. These then appeared to have yielded trophozoites.

Hexamita and Oyster Epizootics.

Certes (1882) was the first to report the occurrence of H. inflata as a commensal in the oyster, O. edulis and since that time there have been a number of other reports of the occurrence of Hexamita species in moribund oysters (Richardson, 1939; Mackin et al., 1952; Drinnan et al., 1961; Stein et al., 1961; Laird, 1961; Scheltema, 1962). In most instances it has not been entirely clear whether the species involved was H. inflata, since most authors carried their identification only to the genus level. In most instances the descriptions have been inadequate; indeed, the descriptions of H. inflata which are available are themselves not sufficient to permit unequivocal identification when it has been reported.

There have been various interpretations of the role of Hexamita in the oyster epizootics with which it has been associated. Richardson (1939) studying the high mortality rate among the oysters of Prince Edward Island, suggested that this flagellate, then referred to as Urophagus, was highly pathogenic. A pathogenic role of Hexamita was also suggested by Mackin et al. (1952) in European and American

oysters on the basis of histopathological studies of the oysters. Trophozoites and bacteria were often abundant in the blood vessels, intestinal and stomach lumina, as well as gonads and adductor muscles of dead oysters. However, since cases of acute hexamitiasis were always accompanied by heavy bacterial infection, the evidence presented to relate the pathological state of the oysters to the mere incidence of Hexamita was certainly not conclusive. Stein et al. (1961) also concluded that Hexamita was a pathogen of O. lurida when they observed a significantly higher mortality of normal oysters exposed to diseased oyster tissues at 6 C. It was determined that although dying oysters not exposed to the tissues were infected with Hexamita, the level of infection was much lower than in oysters which had been exposed. However, Stein et al. apparently did not consider the alternative conclusion: that the diseased tissues contained other agents capable of causing disease in exposed oysters and that the Hexamita infection was secondary.

Our previous studies (Drinnan et al., 1961) on certain phases of Malpeque disease indicated that Hexamita was not the primary causative agent of Malpeque disease; but was one of the earliest invading members of a community of saprophytic organisms occurring in all dying oysters. This conclusion was based on: (1) the widespread occurrence of Hexamita in all gapers; (2) the absence of the flagellate in healthy oysters; and (3) histological studies.

Scheltema (1962) also concluded that Hexamita was not

a primary pathogenic agent in Delaware Bay-oyster mortality. He observed that when a dense population of Hexamita derived from mixed cultures was introduced into a tank of water containing oysters at 6 C, only one out of 30 oysters died at the end of 33 days. Light parasitization was noted in 38% of the surviving oysters. Thus even under exposure to what appears to have been extraordinarily high concentrations of Hexamita, no significant mortality occurred.

A somewhat different interpretation of the role of Hexamita in oyster epizootics has been presented by Laird (1961), as a result of studying the occurrence in association with other microorganisms in oysters of an organism, identified by him as H. inflata. He suggested that under certain unfavourable microecological conditions, the activities of polysaprobes, notably H. inflata, were capable of causing abnormal mortalities in oysters. The author, however, presented no evidence to support his thesis.

While it appears that Hexamita is perhaps not the primary cause of oyster disease, its widespread occurrence in all dying or dead oysters deserves further study in order that its role in the high mortality in these oysters may be understood. It would also be desirable to establish whether one or more than one species of Hexamita is involved in oyster diseases.

Cultivation of Species of Hexamita.

The fact that species of Hexamita are anaerobic, together with their apparently complex nutritional require-

ments has made their cultivation difficult. Bishop (1934) succeeded in culturing the trophozoites of H. gigas from the gut of the horse leech on a diaphasic medium composed of an inspissated serum and an overlay of diluted and inactivated serum in saline. Her cultures, however, were not axenic. By the use of penicillin and streptomycin, Hughes and Zander (1954) grew a bacteria-free culture of H. meleagridis in the allantoic fluid of a developing chick embryo. Scheltema (1962) cultured the trophozoites of species of Hexamita from a shucked oyster suspended in sea water, filtered oyster liquor and antibiotics. However, pure cultures of the organism were not obtained. In the same way, Marteil et al. (1962) succeeded in maintaining agnotobiotic cultures of another unidentified Hexamita derived from Portuguese and French oysters in peptone and sea water enriched with fluid from the stomach or intestine of the oysters.

Thus when our studies began, there were no methods available for the axenic cultivation of Hexamita on artificial media. It was in consideration of the need to fulfill this requirement as prerequisite to the study of its morphology, nutrition and pathogenicity, that the work of this thesis was undertaken.

CHAPTER II

EXPERIMENTAL WORK

Section 1

Isolation and Cultivation of *Hexamita*

Introduction

Upon initiating this work and on the basis of the morphology as described by Laird (1961), the flagellates isolated from the Canadian oyster, *Crassostrea virginica*, were tentatively identified as *H. inflata* (see below).

As an initial step in the study of this organism and its relation to Malpeque disease, attempts were made to isolate and cultivate the flagellate in artificial media in pure culture. In the work reported in this section, the development of a method for the axenic cultivation of the trophozoites of *H. inflata* in a synthetic medium is described.

Materials and Methods

Source of organism --- Gapers known to contain *Hexamita* were used. The meat of the gaper was removed from the shells, washed in several changes of sterile sea water, and placed in a sterile petri plate to allow draining of the excess fluid. The visceral mass of the gaper was scraped with a sterile scalpel and the scrapings were used for inoculation.

Preparation of egg-yolk suspension --- One fresh egg yolk was homogenized with an equal volume of 2.3% NaCl solution. After straining through a double layer of cheesecloth and centrifuging at 5000 G for 15 minutes, the homogenate was adjusted to pH 7.5 with normal NaOH and filter-sterilized through a Seitz filter.

Preparation of beef serum --- A 10% Difco dehydrated beef serum was dissolved in warm (ca. 50 C) 2.3% NaCl solution, centrifuged, the pH adjusted to 7.5 and the solution filter-sterilized as described above.

Media --- The following media were used: nutrient broth (Difco); brain heart infusion (Difco); semifluid thioglycollate broth (British Drug House); Trichosel broth (Baltimore Biological Laboratories) and Hexamita isolation (HI) medium (Table 1). All media were prepared in filtered sea water having a salinity of 25 to 28‰. The basal medium used in the formulation of HI medium was dispensed in 8.5 ml quantities in screw-cap tubes (16x125 mm) and sterilized by autoclaving for 10-15 minutes at 121 C. After autoclaving, either one ml egg-yolk suspension or one ml 10% reconstituted beef serum was added to each tube as well as 0.5 ml of antibiotic solution. The latter was made up to contain in each millilitre: 50,000 units K-penicillin G (Nutritional Biochemical Corporation); 50 mg dehydrostreptomycin sulfate (Nutritional Biochemical Corporation); and 2,000 units mycostatin suspension (Squibb & Sons, Co.). For the preparation of a Hexamita maintenance (HM) medium, the

TABLE 1

Composition of the Isolation Medium (HI) for the Trophozoites of *H. inflata*

Basal Medium	
Peptone	50 mg
Yeast Extract	10 mg
Glucose	20 mg
Cysteine·HCl	2 mg
Agar	7 mg
Filtered Sea Water (S=25-28‰)	8.5 ml
Components Added after Sterilization	
Egg Yolk Suspension or 10% Beef Serum	1 ml
Antibiobic Solution*	0.5 ml
pH 7.5 adjusted with normal NaOH	

* The antibiotic solution contained per millilitre: 50,000 units K-penicillin G, 50 mg dehydrostreptomycin sulfate and 2,000 units mycostatin suspension.

TABLE 2

Composition of Artificial Sea Water

NaCl	22.0	g
KCl	0.6	g
$CaCl_2$	1.0	g
$MgCl_2 \cdot 6H_2O$	2.9	g
Na_2SO_4	0.7	g
$MgSO_4 \cdot 7H_2O$	2.0	g
Na_2HPO_4	0.01	g
KH_2PO_4	0.005	g
$NaHCO_3$	0.03	g
Distilled Water	1000	ml

antibiotic solution was omitted, and the medium was prepared in an artificial sea water (Table 2).

Measurement of growth --- Growth was measured by means of cell counts using a bright line hemocytometer (Spencer-type).

Sterility tests --- Sterility tests were used to determine the extent of microbial contamination. They consisted of : (1) a microscopic examination and (2) inoculation into thioglycollate broth of ca. 0.5 ml of stock culture and incubated at 20 C and 30 C.

Maintenance of stock cultures --- Once axenic cultures were established, they were maintained in HM medium at 17 C, and transferred every 10 days. One-tenth millilitre of inoculum was used.

Results

At the beginning of our work, several unsuccessful attempts were made to isolate Hexamita by inoculating material from gapers into a variety of the common bacteriological media, such as nutrient broth, brain heart infusion, thioglycollate broth and Trichosel broth. Of these, only Trichosel broth, incubated at 5 C but not 20 C, permitted survival of the flagellate population. Since incubation at 5 C permitted survival, this incubation temperature was at first used for our cultivation attempts. When gaper meat harbouring trophozoites was added to any of the above media without adjusting pH, dense trophozoite populations contaminated with bacteria were obtained and could be main-

tained for about 7 days, but serial transfers failed to propagate the flagellate. However, when the suspensions of Hexamita were maintained at a pH in the alkaline range (ca. pH 7.5), dense, active trophozoite populations were maintained for over 3 weeks at 5 C. Despite a longer survival, however, it was impossible to propagate the organism by serial transfers.

In attempting to replace the gaper meat in a medium which would support the growth of mixed cultures of Hexamita, consideration was given to the fact that the trophozoite population was highest in gapers or wintering oysters under conditions in which the phospholipid content was high (Drinnan, personal communication). Therefore inoculations were made into nutrient broth adjusted to pH 7.5 and supplemented with either egg yolk or serum, both of which are known to contain appreciable quantities of phospholipid. When incubation was carried out at 5 C, growth was obtained but always in accompaniment with bacteria. If the initial bacterial contamination was not great, subcultures were then possible.

Having obtained an agnotobiotic culture of Hexamita, attempts to axenize it using antibiotics were made, but at first without success. It appeared that the flagellate required the presence of other microorganisms for growth. Thus when penicillin (100 units/ml) and streptomycin (50 µg/ml) were incorporated into nutrient broth supplemented with egg yolk or serum, monoxenic cultures of

Hexamita contaminated with pink yeast were obtained after several subcultures. It was consistently observed, however, that considerable growth of the yeast always preceded development of the protozoan except when cultivation was carried out in Trichosel broth. Trichosel broth, a medium designed to furnish partially anaerobic or reducing conditions for growth of Trichomonas sp. contains two ingredients, cysteine and a low concentration of agar, lacking in the other media tested. A new medium, HI medium, was therefore devised which contained these ingredients.

Using HI medium (Table 1) it was then possible to obtain axenic cultures of Hexamita. When HI medium was inoculated with gaper scrapings containing trophozoites and incubated at 5 C, axenic cultures of the organism were established after serial transfers at 5-day intervals. One-half millilitre of the culture taken from the lower portion of the tube was used for inoculum. Similarly, axenic cultures of the organism could be obtained within a shorter time if an incubation temperature of 15-20 C was used. In this case, serial transfers were made with 0.1 ml of inocula at 2 to 3- day intervals. Once an axenic culture was obtained, stock cultures of Hexamita were maintained in HM medium at 15-18 C and subcultures at 10-day intervals, or at 3-week intervals if incubated at 5 C. One-tenth millilitre was used for inoculation.

In order to correct for any possible variation in salinity and composition of sea water, artificial sea water

based on the composition of a physiological saline for oysters (Otis, 1942) was used in the maintenance medium.

Seven out of eight trials to isolate the organism from gapers gave positive axenic cultures after five serial transfers in the isolation medium. The ease of obtaining axenic culture was found to depend on the gapers used; those which were heavily infected by many other microorganisms, such as fungi and protozoa, were poor sources of inocula.

Growth in medium containing serum was quite comparable to that supplemented with the egg yolk. In either case, about 10^6 trophozoites per millilitre were obtained after incubation at 5 C for 15 days or 17 C for 5 days.

Section 2

The Morphology of *Hexamita*

Introduction

As indicated previously (p. 1), only two authors, Certes (1882) and Laird (1961), have identified *H. inflata* as the species involved in oysters. The latter author based his conclusions on the observations that (1) the dimensions of the trophozoites overlapped those of *H. nodulosa* (the type species) and *H. inflata* as originally described by Dujardin; and (2) the plump and posteriorly truncate appearance of the trophozoites corresponded closely to that of *H. inflata*. Though we had tentatively identified our organism as *H. inflata*, in the absence of more definitive criteria than those presently available in the literature, it was not possible to make an unequivocal identification. Nor was it possible to determine from the literature the identity of the other species of *Hexamita* which have been described in oysters.

Much of the difficulty is attributable to the generally inadequate descriptions given by the various authors. Therefore it seemed appropriate to include some morphological observations made during the course of our studies. These are described here in the hope that they will add to the number of usable criteria involved in species identification.

Materials and Methods

Four-day-old axenic cultures of the trophozoites of H. inflata originally isolated from an oyster gaper, Cr. virginica, from Bideford River, Prince Edward Island, in April 1961 were used. The cultures were maintained in HM medium and incubated at 17 C as described above (Section 1). Microscopic studies of the trophozoites were carried out by bright field and phase contrast. For some bright field studies, Wright and McNeal stains (Gray, 1954) were used on thin smears. When the Feulgen reaction (Kudo, 1946) was used to stain the nuclear structures, the air-dried smear was hydrolysed in 1N HCl for 5 minutes at 20 C, followed by 5 minutes treatment at 60 C and 20 C. Flagellar arrangement was observed by iodine -vapour fixation of wet smears followed by the protein stain as described by Mazia, Brewer, and Alfert (1953). All slides were mounted in permount or neutral balsam following a series of alcohol dehydrations and a xylene rinse.

Morphological Description

Figure 1 shows a schematic drawing of a trophozoite of H. inflata.

The trophozoites of H. inflata under standard culture conditions in HM medium at 17 C ranged in size from 8-18 μ (average 16 μ) in length by 6-12 μ (average 8 μ) in width. The bilaterally symmetrical body of each trophozoite was oval to pyriform with a slightly pointed anterior, and a slightly blunted or truncate posterior end. The two ovoid

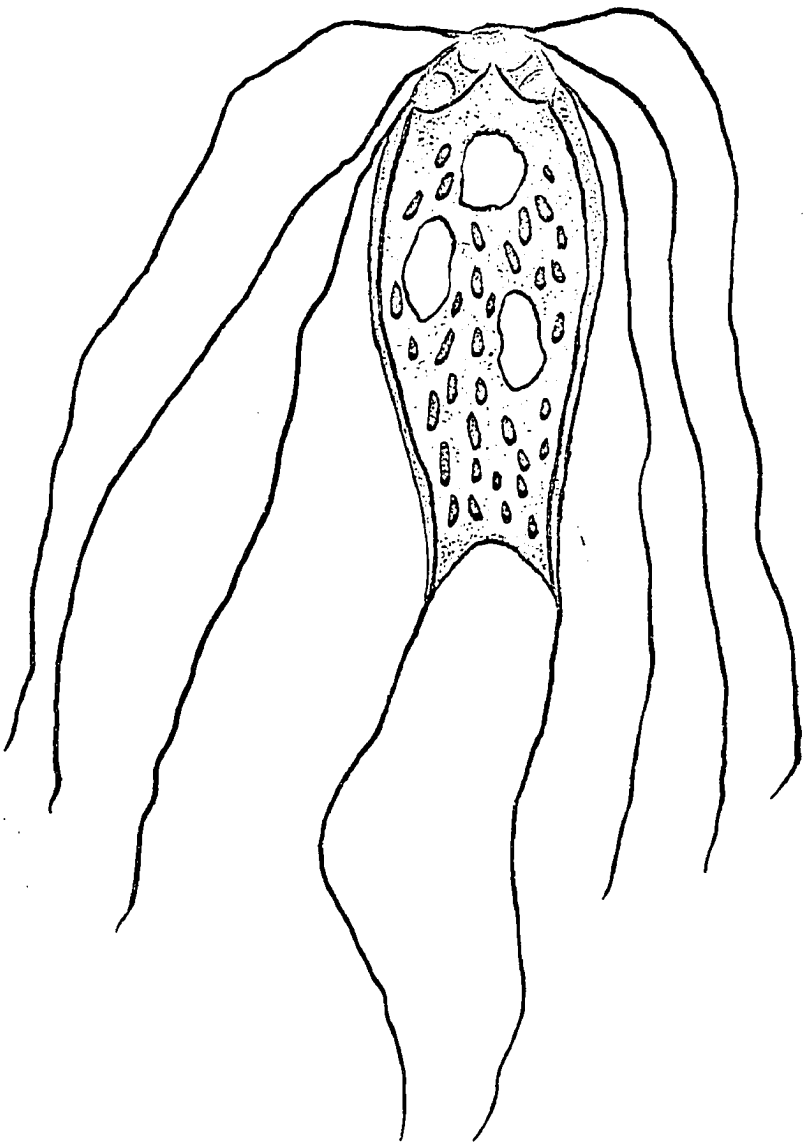

FIGURE 1 A SCHEMATIC DRAWING OF A TROPHOZOITE OF *H. INFLATA*

or spheroid nuclei (2-3 μ by 1-2 μ) were located anteriorly; each contained two large endosomes. Three pairs of anterior flagella were inserted a short distance behind the anterior end. They appeared to enter the nuclei and connect to two axostyles which ran longitudinally along the body. The thin axostyles terminated in two trailing flagella which were ca. 38 μ long or about twice as long as the cell. In some preparations, numerous granules which stained supra-vitally with 10^{-3}M Janus green B and which appeared red or purple when stained with Wright and McNeal stains, were scattered throughout the cytoplasm. In addition to these, within the cytoplasm there were 1-3 vacuoles which often contained inclusion bodies.

The flagellate, under certain conditions which are yet not understood, frequently assumed a spherical form, 9-18 μ in diameter, with or without the full complement of flagella (Figure 2). In these, numerous granules and one to three large vacuoles were occasionally observed. These spherical forms were non-motile, although there was some flagellar movement. The loss of the flagella was generally followed by lysis. When this occurred, only empty spheres remained. These spherical cells of H. inflata were particularly common in smears taken from gapers during the winter.

Multiplication of Hexamita was by longitudinal binary fission only. No cyst formation was ever observed even under a variety of conditions of temperature, pH, redox potential and salinity.

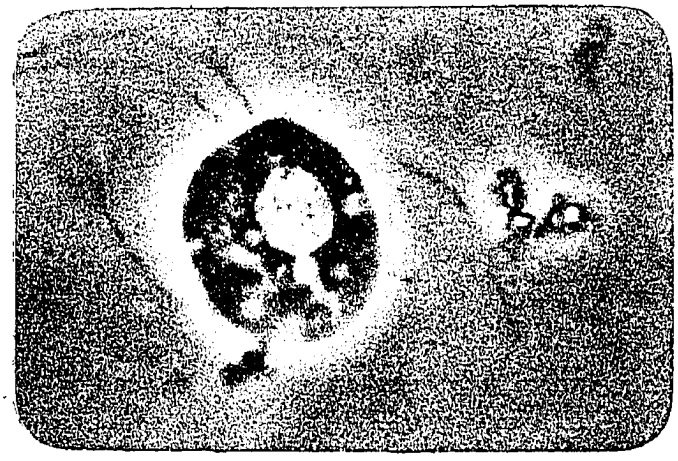

FIGURE 2 A SPHERICAL CELL OF H. INFLATA, PHASE CONTRAST

Section 3

Effect of Physical Factors on Growth

Introduction

The isolation of H. inflata in axenic cultures offered an opportunity to study its growth characteristics under the influence of temperature, pH, redox potential and salinity. Such a study was undertaken with the expectation that it would provide data concerning the physical requirements necessary for artificial culture, and the effects of these factors on morphology, as well as some clues about the conditions permitting the luxuriant population growth characteristically found in dying or dead oysters.

Materials and Methods

Source of organism --- Trophozoites of H. inflata were isolated in axenic cultures from a gaper fished in Bideford River, Prince Edward Island, in September 1962, kept in a bottle and mailed by air to Windsor. Methods for isolation, etc., can be found in section 1.

Media --- HM medium with agar omitted and supplemented with beef serum was used for all the studies in this section. The medium was dispensed in screw-cap tubes (16x125 mm) in 10 ml amounts. For testing the effect of reducing agents, the above basal medium with cysteine omitted was used. Normal solutions of HCl or NaOH were used when necessary, to adjust the pH of the medium before autoclaving. Filtered

and aged sea water (S=28‰) collected from the Bideford River, P.E.I., in 1960, was used for varying the salinity of the medium. The salinity for the diluted sea water was calculated from its specific gravity by means of a hydrographic table (Knudson, 1901).

Inoculation and incubation --- 0.05 ml of a 5-day-old axenic culture of the organism at 17 C in HM medium with 1% beef serum was used for inoculum. All cultures were incubated at 17 C, except when temperature was tested as a variable. Anaerobic cultures were incubated in a Brewer anaerobic jar, evacuated and flushed three times with prepurified nitrogen (Matheson of Canada, Ltd.).

Measurement of growth --- Growth was measured by means of cell counts obtained through the use of a bright line hemocytometer (Spencer-type). Results were expressed as averages of counts from duplicate samples.

Results

Effect of temperature --- Typical results of the temperature experiments are presented in Table 3. Maximum temperature for growth was about 25 C, and no further growth was recorded after 5 days. Growth was quite rapid at about 21 C, but the maximum yields at 21 C and 16.5 C were comparable after 5 and 6 days of incubation, respectively. On the other hand, the growth at 5 C and even lower temperature (above 0 C) was slow and occurred only after a lag of about 2 weeks. But once this lag was overcome, growth occurred until a yield of about 9×10^5 trophozoites

TABLE 3

Effect of Incubation Temperature on Growth of *H. inflata* in HM Medium, pH 7.5

Temperature	Number of Trophozoites/ml X10^5	
	3 Days	5 Days
5.0 ± 1.0 C	1.5*	3.5*
16.5 ± 1.5 C	4.8	8.8
21.0 ± 1.0 C	8.7	10.2
25.0 ± 0.5 C	4.0	5.5
27.0 ± 0.5 C	0	

* Data taken from cultures previously incubated at 5 C.

TABLE 4

Effect of Reducing Agents and Anaerobic Condition on Growth of *H. inflata* in Basal Medium, pH 7.5, 17 C for 5 Days

Addition to Basal Medium	Number of Trophozoites/ml
None	1.8×10^5
Cysteine, 0.02%	9.8×10^5
Thioglycollate, 0.03%	8.2×10^5
Na-sulfoxylate, 0.02%	6.5×10^5
None*	8.8×10^5
Cysteine, 0.02%*	9.5×10^5

* Incubated in Brewer anaerobic jar.

per millilitre was obtained. This required 15 days.

Effect of reducing agents --- The results obtained through the use of various reducing agents are presented in Table 4. Equivalent concentrations of cysteine, thioglycollate and sulfoxylate yielded approximately the same amount of growth as obtained under anaerobic conditions. In the absence of a reducing agent, spherical forms of Hexamita were numerous.

Effect of pH --- The result of experiments to determine the effect of hydrogen-ion concentration on growth is summarized in Figure 3. The maximum trophozoite concentration was obtained at pH 7.0-8.0, although the organism was capable of growing between pH 4.5 and pH 8.5. Tests were not made at hydrogen-ion concentrations higher than pH 8.5. Again, it was also noted that under acidic conditions (ca. pH 4.5), the spherical forms of Hexamita were numerous.

Effect of salinity --- It was found that the trophozoites of H. inflata were able to grow to some extent at all salinity values between 3‰ to 28‰ (Figure 4). There was, however, a sharp decrease in the amount of growth obtained at salinity lower than 20‰. Tests were not made at salinity above 28‰. Vacuolation was observed for trophozoites grown in media of low salinity (3-15‰).

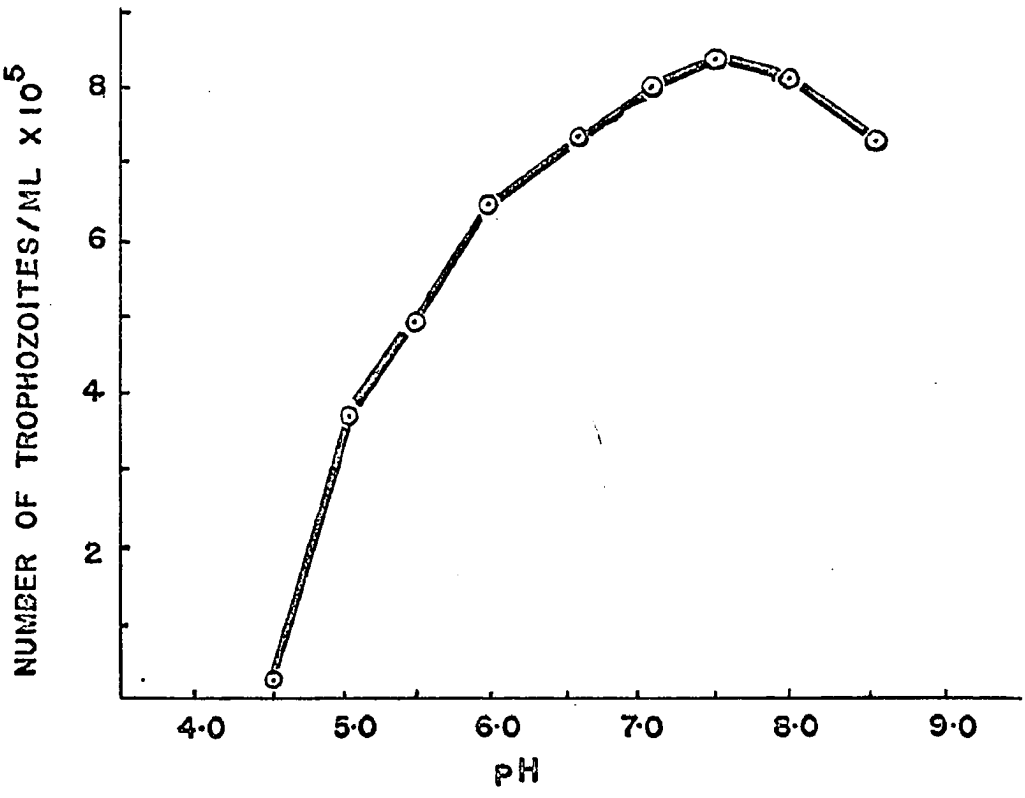

FIGURE 3 THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE GROWTH OF H. INFLATA IN HM MEDIUM, 17° C., AFTER 5 DAYS

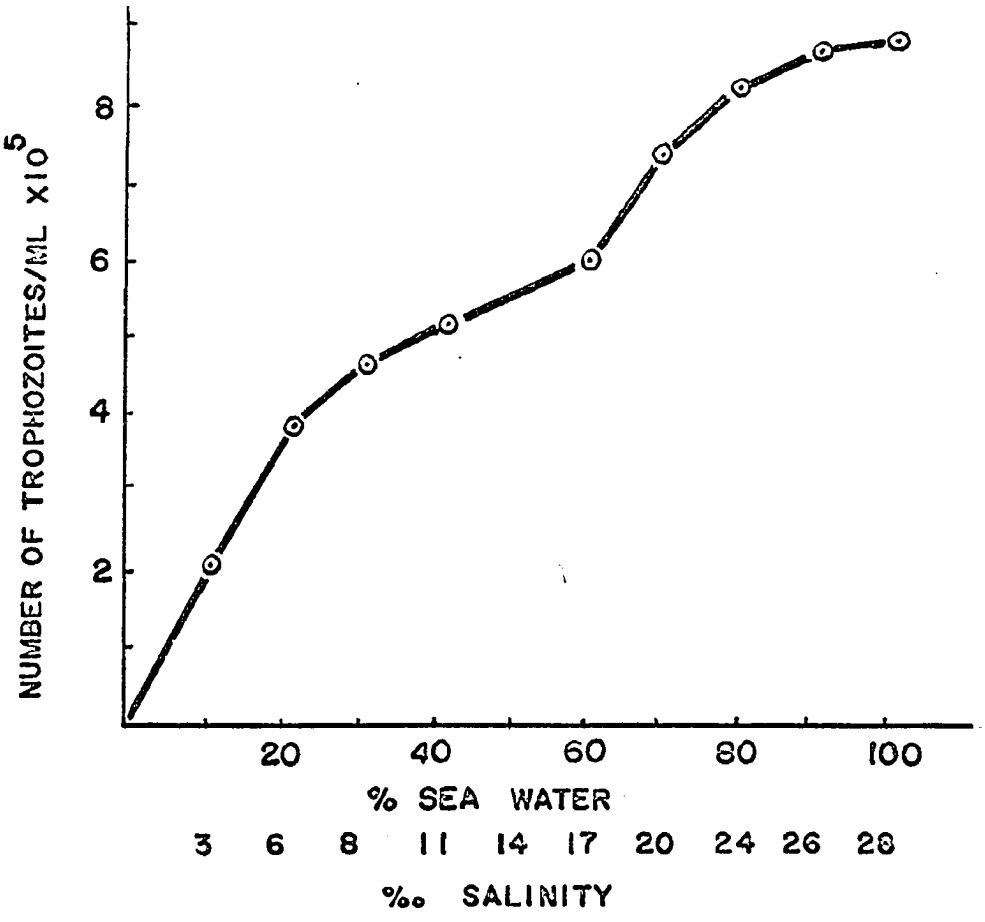

FIGURE 4 THE EFFECT OF SALINITY ON THE GROWTH OF H. INFLATA IN HM MEDIUM, $_{p}$H 7·5, 17° C, AFTER 5 DAYS

Section 4

The Nutritional Studies

Introduction

Having obtained *Hexamita* in axenic culture, it was then possible to carry out studies aimed to a more definitive analysis of the nutritional requirements of the organism than had been previously attempted. It was hoped that these would offer some insight into the metabolism of *Hexamita* as well as permit the synthesis of a simple, more chemically defined medium for use in further studies. This section describes the result of this work.

Materials and Methods

Source of organism --- Axenic cultures of *H. inflata* obtained previously (Section 3) were used.

Chemicals and solutions --- All chemicals, unless otherwise specified, were of reagent grade. Egg lecithin (Nutritional Biochemical Corp.) was dissolved in 95% ethanol at a concentration of 50 mg/ml for use in experiments. Oleic, linoleic and linolenic acids were purchased from Mann Research Laboratories, and stearic acid from Fisher Scientific Company. A stable fatty acid suspension of each of these acids was prepared as described by Miller and Johnson (1960). L-Amino acids were purchased from Mann Research Laboratories. An amino acid solution (19A) (Seaman, 1963) containing 19 amino acids, and another (13A)

containing 13 amino acids (Table 5) were used. A mixture of purines and pyrimidines (Seaman, 1963) and a trace metal mix (Visniac, 1961) were also used in some experiments. Tween 80 was a product of the Atlas Powder Company.

Media used --- The basal medium (Table 6) used was a modified HM medium, in which yeast extract was replaced by a vitamin solution (Table 7). The latter was prepared in a 100X concentration and filter-sterilized by means of a sintered glass filter (UF grade). One-tenth millilitre of the vitamin solution was added to 10 ml autoclaved and cooled medium. A medium designated as HF (Table 8) was used for amino acid experiments. The medium was dispensed in 10 ml amounts in screw-cap tubes (16x125 mm) and autoclaved at 121 C for 10-15 minutes.

Fractionation of egg yolk --- The egg yolks from supermarket "grade A" eggs were extracted with ether at 6 C for 24 hours. The ether-insoluble fraction was dried at 30 C overnight. The ether-soluble fraction was divided into equal portions as follows: (1) One portion was injected into an equal volume of cold acetone to obtain the lecithin containing precipitate. This was collected by centrifugation and dried at 30 C for 6 hours. The supernatant was evaporated to dryness at 45 C in a Spinco evaporator. (2) The other portion of the ether-soluble fraction was evaporated to dryness in a Spinco evaporator. A volume of sterile artificial sea water, adjusted to pH 7.5, equal to twice the volume of the egg yolks used, was added to each fraction.

TABLE 5

Composition of a 10X Solution of 13 Amino Acids (13A) present at the Equivalent of 0.5% Bacto Peptone

L- Arginine	400	mg
L- Aspartic Acid	300	mg
L- Glutamic Acid	550	mg
Glycine	1150	mg
L- Histidine	50	mg
L- Isoleucine	100	mg
L- Lysine	225	mg
L- Methionine	50	mg
L- Phenylalanine	100	mg
L- Threonine	75	mg
L- Tyrosine	50	mg
Distilled Water	100	ml
pH 7.5 adjusted with normal NaOH		

TABLE 6

Composotion of Basal Medium (BM) used for Nutritional Studies of *H. inflata*

Peptone	500	mg
Glucose	200	mg
Cysteine·HCl	20	mg
Vitamin Solution	1	ml
Artificial Sea Water	99	ml
pH 7.5 adjusted with normal NaOH		

TABLE 7

Composition of a 100X Vitamin Solution (after McCurdy, 1963)

Thiamine	5	mg
Riboflavin	35	mg
Pyridoxal·HCl	3.5	mg
Ca- Pantothenate	10	mg
Nicotinic Acid	3.5	mg
Biotin	0.03	mg
Vitamin B_{12}	0.03	mg
p- Aminobenzoic Acid	0.25	mg
Inositol	500	mg
Choline	20	mg
Folic Acid	0.05	mg
Distilled Water	100	ml

TABLE 8

Composition of HF Medium used for Amino Acid Experiments

Glucose	200 mg
Cysteine•HCl	20 mg
Linoleic Acid	4 mg
Vitamin Solution	1 ml
Artificial Sea Water	99 ml
pH 7.5 adjusted with normal NaOH	

The mixture was vigorously shaken until a uniform suspension was obtained. One millilitre of each of the suspensions was used for every 9 ml of sterile basal medium. As a precaution against possible contamination, aseptic technique was used throughout these operations.

Measurement of growth --- In order to obtain an estimate of growth, each culture was sampled with a sterile pasteur pipette from which one drop was carefully deposited on a slide. A cover glass (# 1) was applied to the drop and the sample observed under a magnification of 400X. Cell counts were made from five random fields. Overall examinations of slides generally showed a fairly homogeneous distribution of trophozoites in each preparation. The results were scored as 0, 1+, 2+ and 3+ corresponding to 0, 1-15, 16-25 and >26 trophozoites per field respectively. For more accurate determination of trophozoite number, a bright line hemocytometer (Spencer-type) was used.

Assay procedures --- The medium was inoculated with 0.1 ml of 5-day-old culture of the organism in HM medium containing egg-yolk suspension, and then incubated at 17 C. At weekly intervals, transfers were made of the cultures carried on media containing the various supplements. A supplement was considered active if it supported growth through 5 such transfers. For each transfers, 0.1 ml of inoculum was used.

Results

Development of a basal medium --- For the study of

growth factors, a less complex medium than HM was desired. Of the components present in HM medium, yeast extract was concluded to be non-essential for the growth of H. inflata, on the basis that its omission from the medium did not affect the yields obtained even after several serial transfers. However, vitamin solution was added in its place in order as much as possible, to rule out vitamin deficiency as the cause of any alteration of growth, as resulting from the omission or replacement of other ingredients.

Replacement of egg yolk --- The omission of egg-yolk suspension from the medium completely inhibited growth as shown in Table 9. In an attempt to determine the nature of the growth promoting component or components, the egg yolk was fractionated with ether. It was found that only the ether-soluble fraction contained the substance or substances necessary for growth. When this ether-soluble portion was further fractionated with acetone, only the acetone precipitable fraction, which presumably contained lecithin, supported limited growth. However, both the acetone-soluble and acetone-insoluble fractions were required for continued growth after 5 serial transfers. In a subsequent experiment, it was found that the growth activity of the ether-soluble fraction, and the egg-yolk suspension could be replaced by egg lecithin at a concentration of 150 µg/ml. Thus it appeared that the active component of the egg yolk might have been lecithin or a substance or substances derived from it, such as fatty acids. Consequently, a number of

TABLE 9

Growth Response of H. inflata in Basal Medium (BM) with Various Supplements at 17 C for 7 Days

Fraction No. (Fr)	Supplements Added to BM	Number of Subcultures					
		1	2	3	4	5	6
0	None	2+	1+	0			
1	Egg Yolk	3+	3+	3+	3+	3+	3+
2	Ether-soluble Fr 1	3+	3+	3+	2+	3+	3+
3	Ether-insoluble Fr 1	2+	1+	0			
4	Acetone-precipitated Fr 2	3+	2+	2+	1+	0	
5	Acetone-soluble Fr 2	2+	1+	1+	0		
6	Fr 4 + Fr 5	3+	2+	3+	2+	2+	3+
-	Egg Lecithin 150 µg/ml	3+	3+	2+	3+	2+	2+

TABLE 10

Growth Response of H. inflata in Lipid containing Basal Medium after 7 Days at 17 C

Addition to Basal Medium	Concentration µg/ml	Number of Subcultures					
		1	2	3	4	5	6
None		2+	1+	0			
Egg Yolk		3+	3+	3+	3+	3+	3+
Oleic Acid	30	2+	2+	1+	2+	1+	1+
	120	2+	1+	1+	0		
	300	0					
Linoleic Acid	15	2+	2+	1+	2+	2+	2+
	150	1+	1+	1+	1+	1+	1+
Linolenic Acid	20	2+	1+	1+	2+	1+	1+
	80	2+	1+	0			
	120	0					
Stearic Acid*	20	1+	1+	0			
	200	2+	2+	1+	1+	1+	1+

* After 15 days of incubation.

unsaturated fatty acids, including oleic, linoleic and linolenic acid, and a saturated fatty acid, stearic acid, were tested for their egg-yolk-replacing activity. All the unsaturated fatty acids tested were found to support growth in the basal medium, as shown in Table 10; but the yield of the organism in the linoleic acid medium was higher compared to that in either oleic or linolenic acid. It was also found that concentrations of the unsaturated acids greater than ca. 100 µg/ml were inhibitory to the growth of Hexamita. On the other hand, the saturated fatty acid tested had only low activity, and only at a high concentration and after a prolonged incubation was the activity of stearic acid comparable to that of linoleic acid.

In Figure 5, the growth of Hexamita in the medium containing linoleic acid and linoleic supplemented with Tween 80 as a dispersing agent is compared to that obtained on the egg-yolk medium. It was found that although the final yields of cultures in all media were comparable, the growth rate of the organism in a fatty-acid-containing medium was smaller compared to that of the egg-yolk medium. Furthermore, the growth of the organism in a fatty-acid-containing medium was preceded by a longer lag. Attempts to reduce the lag by the addition of surfactants such as Tween 80 (1 mg/ml) and sodium deoxycholate (0.01%), doubling the concentration of vitamins, and the addition of trace metals, were not successful.

Replacement of peptone --- In an attempt to replace

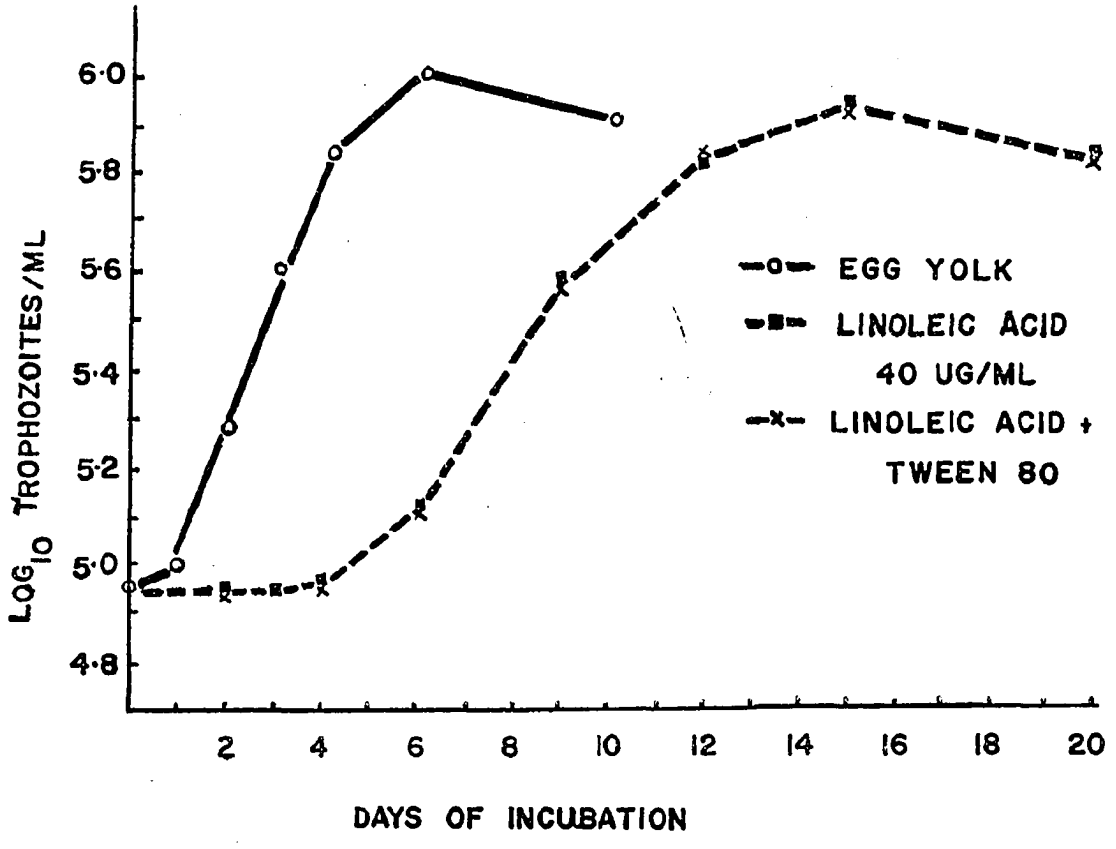

FIGURE 5 GROWTH CURVES OF H. INFLATA IN HM MEDIUM AND BASAL MEDIUM SUPPLEMENTED WITH LINOLEIC ACID

peptone in a linoleic acid containing medium (HF medium), each of the amino acid solutions, 19A and 13 A, was tested but without success, as shown in Table 11. Moreover, as can be seen in the table, no stimulation of growth was obtained when a mixture of purines and pyrimidines was used to supplement the amino acid mixtures. Further attempts to replace the peptone by modifying the amino acid concentration were unsuccessful, and experimentation was discontinued.

TABLE 11

Growth Response of H. inflata in HF Medium containing Amino Acids after 7 Days at 17 C

Addition to HF Medium	Number of Subcultures					
	1	2	3	4	5	6
None	2+	1+	0			
Peptone, 0.5%	2+	2+	1+	2+	2+	2+
19 Amino Acids (19A)	2+	1+	0			
19A + Purines & Pyrimidines	2+	1+	0			
13 Amino Acids (13A)	2+	1+	0			
13A + Purines & Pyrimidines	2+	1+	0			

CHAPTER III

DISCUSSION AND CONCLUSIONS

In this thesis, the first successful attempt to cultivate the trophozoites of *H. inflata* in axenic culture in an artificial medium is reported. This was accomplished using organisms obtained from the oyster, *Cr. virginica*, through serial transfers in a medium containing peptone, glucose, yeast extract, cysteine and egg-yolk suspension or serum prepared in artificial sea water. The antibiotics penicillin, streptomycin and mycostatin were used to free the flagellate from contaminating microorganisms. The use of egg yolk or serum and cysteine as well as antibiotics in deep culture were the essential conditions for obtaining the axenic culture of the organism. The ease and reproducibility of this technique made it possible to undertake further studies on the organism not attempted previously. Since this work was first reported (Khouw, 1962), Uzmann and Hayduk (1963) have also reported a similar method for the *in vitro* cultivation of *H. salmonis* in medium 199, supplemented with human cord serum, lactalbumin hydrolysate, antibiotics and buffered saline maintained in anaerobic conditions.

The trophozoites of *H. inflata* under natural conditions live in close association with dying or dead oysters, which commonly occur during the winter when water temperature is

about 4 C. At this temperature range, the oysters remain closed (Henderson, 1961) and conditions within these closed oysters were found to be acidic (ca. pH 5) and anaerobic or reducing. Although the conditions of temperature, salinity and pH obtaining in the oysters do not correspond to the optima for the growth of Hexamita as determined in our experiments, they do fall within the experimentally-determined ranges for growth. It would seem that the important physical factor operating in the oyster is the reducing or anaerobic environment.

In the nutritional work, our primary effort was directed to replacing the egg-yolk component of the maintenance medium. As a result of fractionating the egg yolk with lipid solvents and testing the effect of various fractions, it was demonstrated that the function of the egg yolk was likely that of supplying some unsaturated fatty acids. In any event, the egg yolk was replaceable by linoleic acid and to some extent by oleic or linolenic acid. Despite the apparent activity of the saturated fatty acid tested, namely, stearic acid, the high concentrations required suggested that an unsaturated fatty acid contaminant might have been responsible for its growth-promoting properties.

The nutritional role of peptone was not completely studied. It was assumed that the role of this casein digest was to supply amino acids required for growth. Similar difficulties have been encountered in other nutritional studies of other protozoa. For example, in

Tetrahymena, Dewey and Kidder (1958) reported a toxic effect resulting from an improper balance of amino acids in the medium used to cultivate this ciliate. Holz et al. (1961), working with T. paravorax, also reported that a specific peptone fraction such as sterol might be important in detoxifying linoleic acid. On the other hand, Weiss and Ball (1947), working on Tritrichomonas foetus, demonstrated a requirement for a streptogenin-like factor present in casein hydrolysate. Thus there are a number of possible explanations for our failure to replace the peptone in our very preliminary experiments.

The simple structure of the trophozoite of H. inflata has contributed to the difficulty of species identification. The results of our morphological studies are similar to those described by Laird (1961). Most studies of the organism have presented only the size and shape of the trophozoite, but it is doubtful if these criteria can be confidently used for adequate identification. Moreover, it has been pointed out by Wenrich (1935) that there existed a remarkable uniformity in morphology among species of Hexamita found in hosts that were widely separated taxonomically, therefore, it seems desirable that other criteria be used for more definitive species identification.

Species identification among the protozoa is sometimes made possible when cysts or other forms constitute parts of the life-cycle of an organism. In spite of the fact that cysts were reported for some species of Hexamita (Davis,

1925; Schmit, 1920; Slavin and Wilson, 1960), their absence under various conditions of temperature, pH, redox potential and salinity argued against their formation by H. inflata. However, Mackin et al. (1952) reported the occurrence of binucleate, thin-wall cysts for the species of Hexamita from O. edulis and Cr. virginica. Since their histological observations were based on materials taken from oyster tissues, the origin and identity of these cysts becomes doubtful. It is also interesting to note in this respect, that under certain unfavourable conditions such as a low pH or oxidizing environment, H. inflata could assume a spherical form, with or without flagella. It is possible that this structural form could have been mistaken for a cyst.

The importance of the flagellar structure for taxonomic purposes in the flagellated protozoa is well known. In the most recent studies on Trichomonas vaginalis (Honigberg and King, 1964) and T. gallinae (Abraham and Honigberg, 1964), these two closely similar organisms were differentiated, among other morphological characteristics, on the basis of the structure of their flagellar units, which were demonstrable by means of the protagol-silver staining method. In our preliminary morphological studies of H. inflata, no similar work has been attempted. However, it is doubtful if this approach can be used to any useful extent in the case of Hexamita, which is characterized by a simple and relatively undeveloped flagellar unit. On the

other hand, since the flagellate can now be cultivated in axenic cultures, additional parameters based on physiological characteristics such as nutritional requirements for growth, may also be advantageously used.

There have been no other previous reports of nutritional studies of Hexamita with which to compare our results. There have, however, been a number of studies on the related trichomonads, and it is of interest to compare the nutrition of these two anaerobic flagellates. The trichomonads are morphologically more complex than Hexamita, and are characterized by the presence of an undulating membrane and some other structural components which are absent in Hexamita. These components may include costa, pelta and a well-developed axostyle protruding beyond the posterior end (Honigberg, 1963). Therefore, the trichomonad would seem to be phylogenetically more advanced, although it is conceivable that the simplicity of Hexamita is due to reduction of structures. On the basis of the concept developed by Lwoff (1951), who considered the requirement for growth factors in an organism as the consequence of losses of functions, it would be expected that the nutritional requirements of the trichomonads would be more complex than those of Hexamita if the former flagellates were phylogenetically more advanced. Indeed this is the case, as seen in a comparison of the results of our studies on Hexamita with the nutritional requirements of trichomonads reported in the literature. Thus, although both flagellates have

similar requirements for linoleic acid and a casein hydrolysate (Sprince and Kupferberg, 1947), the trichomonads also require some saturated fatty acids (Shorb and Lund, 1959) and, in addition, many require sterols (Cailleau, 1936; Lund and Shorb, 1962). Although it is premature to carry this kind of speculation too far, it would be interesting to know whether studies of other species of Hexamita would yield results consistent with Lwoff's hypothesis.

CHAPTER IV

SUMMARY

1. An axenic culture method for the isolation and cultivation of the trophozoites of H. inflata was developed. The medium prepared in sea water (S=25-28‰) was composed of peptone, glucose, yeast extract, cysteine, egg-yolk suspension or beef serum and antibiotics.

2. A brief morphological description of H. inflata was attempted. No cyst formation in culture was observed, but the formation of a spherical cell, with or without flagella, was noted in culture under certain conditions.

3. The effects of physical factors on growth in culture were determined. The flagellate was capable of growing under a wide range of temperature (5 C to 25 C), hydrogen-ion concentration (pH 4.5 to 8.5), and salinity (3 to 28‰), and required a reducing or anaerobic environment.

4. The requirement, for growth, of an egg-yolk suspension was partially satisfied by unsaturated fatty acids. Attempts to replace peptone with mixtures of amino acids were not successful. However, a partially defined medium containing vitamins, an unsaturated fatty acid, glucose, cysteine, peptone and salts was formulated.

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