The culture of some holotrichous ciliates.

By

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(With 1 figure in the text.)

Introduction.

The organisms investigated were *Colpidium campylum*, *Glaucoma scintillans*, *Loxocephalus granulosus*, and *Colpidium colpoda*. They are members of the family Frontonidae, which is included in the suborder Hymenostomata. They belong to the group of continuous feeders designated by German authors "Schleudern" in contrast to the "Schlingern", occasional feeders, whose mouths are usually shut (Gymnostomata).

In the treatment which follows, culture methods and results will be described for each organism, in the order given above, in which also they were investigated.

Some generalizations, with special reference to relations to the hay infusion cycle, are rendered in the Discussion.

The series of investigations here recorded, involving a variety both of organisms and of methods, is difficult to render in a clear and concise fashion. It has seemed advisable, therefore, to offer briefly an historical review of some former investigations on the culture of these ciliates. This can at best be only fragmentary since our knowledge of the cultural requirements of the ciliated Protozoa is at present altogether rudimentary; indeed, in the case of *Loxocephalus* there is nothing whatever to report upon.

Colpidium campylum.

Oehler, in 1919, announced the culture of an organism, identified by Clara Hamburger as *Colpidium colpoda*, on suspensions of *Bacillus subtilis*. He was unable to obtain growth in pure culture consisting of dead *Bact. coli*.

In 1920 he published further results. This ciliate was found to grow on living *Bact. fecalis alkaligenes*, *Bact. pyocyaneus*, *Staphylococcus aureus*, *Sarcina lutea*, and *Spirillum volutans*, and he concluded that it will probably grow on almost any bacterium. He found that dead bacteria will not support growth, but that *Bact. coli* + 1 % peptone-glucose gave excellent growth. Pure culture was not achieved.

In 1922 BRESSLAU corrected the name of OEHLER'S ciliate to *C. campylum*, describing it and *C. colpoda*. The paper leaves little room for confusion; the two species may indeed be distinguished with the naked eye.

In 1920 PETERS announced the sterile culture of *Paramecium* in a preliminary paper.

In 1921 his organism, identified by DOBELL as *C. colpoda*, was grown on a series of media including .06 % ammonium glycerophosphate. He reported the presence of rod-like bodies which he could not cultivate and which he decided were discarded cilia or patches of cilia.

In 1929 experiments are reported on this ciliate grown in a sterile medium containing sterilized yeast. Growth required the presence of solid matter in the medium.

Judging from its morphology and physiology (except for its requirement of particulate food in the form of autoclaved yeast cells) as described in the literature, this organism is *C. campylum* not *C. colpoda*.

CUTLER and CRUMP (1923—1924) used PETERS' *Colpidium*.

BUTTERFIELD (1929 and 1931) reports the sterile culture of "*Colpidium*" in .5 % each of peptone-glucose. They increase to a maximum of about 50,000 per cc. He further states that it flourishes on *Bact. aerogenes* in dilute peptone-glucose. Judging from the volume, given as 32,000 cubic microns, the organism is *C. campylum*. LWOFF (1932, p. 62) ventures the suggestion that it is *Glaucoma piriformis:* "Ces auteurs ignorent également tous les travaux qui précédent le leur. Peut-être ont-ils cultivé *Glaucoma piriformis?*"

Mr. R. M. BOND [1]), using my *C. campylum* on yeast extract + dextrose was able to obtain 200,000 organisms per cc.

Glaucoma scintillans.

In general this ciliate, like *C. campylum*, will grow on living, but not on dead, bacteria. CHATTON (1925) has grown *G. scintillans*

[1]) Personal communication.

on ammonium lactate-glucose + *Pseud. fluorescens* for more than six years, obtaining, on the fifth day of a subculture, 12 to 15,000 organisms per cc. He was able to maintain the ciliate on dead bacteria (1929), however, by allowing them to grow in the nutritive system before killing by means of heat. I quote from page 1315: "D'après nôtre expérience, les Ciliés ne s'entretiennent pas sur les bactéries tuées dans leurs cultures mêmes (sur gélose par example) et transportées dans le milieu neuf pour Infusoires. Il faut que ce milieu ait été préablement travaillé par les bactéries".

The sterile culture of *Glaucoma scintillans* has not been achieved up to this time.

It is not entirely out of place to briefly report here the sterile culture of *Glaucoma piriformis.* In 1923 Lwoff announced the sterile culture of *Colpidium colpoda* on peptone broth. A 1924 paper reports the sterile culture of *Glaucoma pyriformis*, and, we are told in his monograph (1932), constitutes a redress of the 1923 error.

Colpidium colpoda.

The name *Colpidium colpoda* appears to have in the literature generally quite an unrestricted usage. In reading the papers just reviewed, one comes to wonder whether any of these investigators, excepting Bresslau, actually identified and worked with this ciliate. Bresslau's paper of 1923 is a systematic treatment in which the two species *C. colpoda* and *C. campylum* are described and clearly distinguished. The former species is, according to this author, relatively rare.

It appears likely that in at least two of the papers which are of some interest with reference to culture, however, the investigations had to do with *C. colpoda.* Chatton (1923) studying conjugation in *Glaucoma* and other forms, states that he has never observed it in *C. colpoda.* This agrees with my experience, for I have seen *C. campylum* conjugate in certain spinach cultures, but never *C. colpoda*, under a great variety of conditions. Chatton (1925 a) notes the formation of chains of cells ("L'action dystomigène") both in *C. campylum* and *C. colpoda* by one specific strain of Coli bacteria. It thus becomes apparent that we know practically nothing about the culture of *C. colpoda.*

Experimental: *Colpidium campylum.*

Sources. This ciliate is one of the commonest of the fresh water Protozoa. It appears in the early stages of the usual infusions of hay, wheat, or lettuce if they are inoculated with pond water.

Enrichment. The first method used was that suggested by BĚLAŘ (1928, p. 818) for *Colpoda Steini*. Three grams of fresh spinach were ground up in a mortar and added, without heating, to 100 cc. tap water. The *Colpidia* flourish on the vegetable particles, becoming quite green and opaque. Those cultures which did not turn strongly acid or alkaline, sub-cultured nicely.

BĚLAŘ (p. 819) quotes OEHLER's culture-technique for *Colpidium campylum*: .1—1. % peptone-glucose + *Bact. subtilis*. He further states that small forms, such as *Colpidium*, *Colpoda*, *Chilodon*, may be cultivated on alkaline "KNOP-agar", which is simply agar made up with dilute alkaline KNOP's solution. This was true of the agar used by BĚLAŘ at Berlin, but is not a satisfactory technique using standard brands of agar [1]). Doubtless a substitution of very dilute beef-extract for the KNOP would correct the difficulty, but this was not tried because of the discovery of a better method of enrichment.

Growth on suspensions of bacteria: The phrase „suspension of bacteria" will be used to designate only those non-nutritive, inorganic systems containing bacteria indroduced from an extraneous source. Ten *Colpidia* were placed in each of a series of watch-glasses or Protozoa Isolation Dishes [2]), each containing 1 cc. of tap-water. The bacteria to be tested were introduced into each dish in increasing amounts, such that the first dish constituted a dilute suspension, and the last an opaque one. This may be done by scraping a pure culture of the bacteria from the surface of a nutritive agar plate with a platinum needle. After introduction, with, perhaps, gentle agitation to remove the bacteria, the needle is, of course, to be flamed before further use.

In the following list of bacteria thus tried, the unidentified forms constitute pure colonies plated out from spinach cultures supporting large numbers of healthy *Colpidium campylum*.

Bacterium	Growth
Yellow (gram negative)	large
White „ „	large
Gray „ „	large
Light yellow	large
Pink	slight
Bacillus subtilis	slight
Bact. coli	very large

[1]) This technique was tired under Dr. BĚLAŘ's direction at the California Institute of Technology. The agar used by him at Berlin was apparently quite impure, thus supplying the dilute nutrient required for suitable bacterial growth.

[2]) Watch Glasses No. 9851, ARTHUR H. THOMAS Co., Philadelphia.

Bacterium	Growth
Bact. prodigiosum (white strain)	very large
Bact. prodigiosum (red strain)	large
Pseudomonas fluorescens	fair
Bact. pyoseptica	slight
Bact. kiliensis	fair
Bact. plymouthensis	fair

The unidentified forms were probably fresh water pseudomonads (Van Niel).

A consistent difference between growth on colored and colorless *Bact. prodigiosum* was observed, the colorless being superior. No experiments testing the extent or nature of this relation were made, but two correlations may be noted. Under the conditions of diffuse light in which all my experiments were carried out, it is known that the red strain tends to die. The second correlation concerns an interesting paper by Chatton (1927). In working with thick suspensions of chromogenic bacteria he describes a cytolytic action (on *Glaucoma scintillans*) which, if consummated within a period of 3—10 minutes, may be resolved into four phases which concern mobility, activity of cilia, contour, mitochondria, granulation of the cytoplasm, etc. In studying 67 species of bacteria he distinguishes 5 graded groups, the fifth of which causes cytolysis within 10 minutes and includes *Bact. prodigiosus*, *Bact. fluorescens*, and a very chromogenic *Bact. pyocyaneus*.

The behaviour in *Bact. coli* differs somewhat from the other suspensions in that a reduction in the number of bacteria is soon appreciable. The suspension tends to be cleared. Possibly this is due to a concentration of the bacteria in one part of the dish in consequence of subsidence. This tendency to clear the system is exactly the behaviour of *Colpoda cucullus* in suspensions of *Pseud. fluorescens* used by Barker (1931).

One other peculiarity of *Bact. coli* in suspension is to be noted. The more concentrated dishes showed a lag in the increase of the *Colpidia* with indications of injury, while later experiments with very opaque suspensions demonstrated the possibility of killing this resistant ciliate through excessive numbers of this bacterium.

Since suspensions of *Bact. coli* are injurious, in my experience, to most ciliates, they may be used as enrichment cultures for *C. campylum*. *Bact. coli* is a satisfactory food organism for this ciliate.

Sterile culture.

My attempts to culture *C. campylum* in the summer of 1930, while still ignorant of PETERS', 1929 paper, paralleled in such a striking manner the experience of PETERS that it was considered worth reporting briefly.

The ammonium lactate-glucose medium was made up according to PETERS. The *Colpidia* were sterilized by washing in a manner essentially that of PETERS. Ten tubes containing 10 cc. of medium each were sterilized (autoclave: 15 lbs. for 15 min.). Six of these tubes were inoculated with *Colpidia*. There was no immediate growth. After 13 days an inspection showed extensive growth in 5 tubes, the sixth remaining perfectly clear, like the 4 uninoculated tubes. Small volumes of these tubes were plated out on nutritive agar, others into yeast extract and incubated at 20, 25, and 37° C. No growth of bacteria was to be appreciated. The successful cultures of *Colpidium* were sub-cultured in an extensive series of tubes of ammonium lactate-glucose with similar growth.

Prof. C. B. VAN NIEL of the HOPKINS Marine Station very generously helped me at this point, examining a sample of medium in the region of the meniscus under the critically illuminated field of an oil-immersion objective. After close observation he was able to demonstrate a number of rod-like bodies which might well be taken for the "discarded cilia" of *C. campylum*. They typically adhered to the cover-slip or slide and were found in patches. Dr. VAN NIEL was able to culture these organisms on nutritive agar plates, obtaining slight growth after three days of incubation at 25° C.

PETERS had reported (1920) the accumulation of certain minute bodies in his culture-tubes which could not be cultivated. He considered that they might represent discarded cilia or patches of cilia from the *Colpidia*. In the light of the above experience it appears more probable that the ammonium lactate-glucose constitutes a satisfactory culture medium for these bodies, which are in reality slow-growing fresh-water bacteria.

It is a striking characteristic of PETERS' media that they are quite dilute. In this connection it is interesting to note BUTTERFIELD'S (1929) comment to the effect that bacteria in polluted water may act as concentrators for bacteria-feeding Protozoa, of the dilute nutritive substances in solution.

It was concluded that *C. campylum* will not live on solutions containing inorganic sources of nitrogen only.

This is of considerable general interest, as is indicated in the last edition of BAYLISS' General Physiology (1927, p. 249): "It has been generally believed that the animal organism, even in its lowest forms, the protozoa, is satisfied with nothing less complex that glucose as a source of carbon. Recent experiments described by PETERS cast some doubt, however, on the general application of this belief. He has succeeded in growing the protozoan, *Colpidium colpoda*, in pure culture in inorganic media to which sources of carbon containing not less than three carbon atoms in the molecule have been added. . . It was believed at one time that animals, at all events higher ones, required nitrogen in the form of more or less complex proteins. . . *Colpidium colpoda* can, however, as PETERS has shown, grow and reproduce normally in a medium free from other visible organisms, and containing ammonium glycerophosphate and salts of calcium, magnesium and sodium chloride."

Colpidium campylum is thus strictly an animal, whose nutritive requirements may be summed up by quoting LWOFF's (1932, p. 145) conclusions for his *Glaucoma piriformis*: "D'autres comme *Glaucoma piriformis* sont incapables de se multiplier dans des milieux ou l'aliment azoté est un sel d'ammonium, un mélange d'acides aminés ou un protide (muscle) ayant subi une digestion pepsique, trypsique et ereptique. Il leur faut des solutions de peptones complexes, résultant de l'hydrolyse ménagée, pepsique ou trypsique, de protides animaux, muscle, ou végétaux, graine d'arachide: métatrophie."

At a later time, following the discovery of a source of *C. colpoda*, ammonium lactate-glucose was again tried on this animal, without the slightest success. The next experiments on *C. campylum* concerned its growth on yeast extract [1]). More organisms were washed, this time very carefully, then introduced into some tubes containing 10 cc. each of yeast extract. The details of the method of successful sterilization by washing which has been perfected in consequence of much use will be described following the present conclusions concerning *C. campylum*.

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[1]) Preparation: 1 lb. pressed yeast (baker's yeast) is well ground in small amounts at a time in 500 cc. tap water. Place in 1 l. flask, plug with cotton, and incubate at 50° for 24 hours. Boil and add NaOH to pH 7 (dilute sample 1 : 10). Filter: the first portions must be filtered over; this process requires about 12 hours. Place in flasks and sterilize 15 lbs. for 15 minutes. This is called yeast autolysate; yeast extract is 10% yeast autolyate.

Three tubes containing 10 cc. each of yeast extract were inoculated with one carefully washed *Colpidium* apiece. Five more tubes were inoculated with several *Colpidia* each. Only one of the isolation-tubes showed growth, the others remaining clear, while of the tubes containing several *Colpidia* two showed growth of this organism, while the others manifested an immediate and intense overgrowth of bacteria. The culture derived from the single *Colpidium* was transferred by means of a sterile capillary pipette to each of 36 tubes containing (4 of each) Yeast Autolysate in the following concentrations: sec, 1:1, 1:2, 1:3, 1:5, 1:10, 1:20, 1:50, and 1:100. Growth was nil in the pure and 1:1 dilution, slight in the 1:2, excellent in all the others, being most rapid in the 1:5 dilution, and less rapid according to the decreasing concentration in the others. Dr. VAN NIEL pronounced these perfectly sterile in consequence of careful tests and they have been maintained in continuous culture by him at the HOPKINS Marine Station. The author has prepared similar sterile cultures of this species of *Colpidium* on four additional occasions, using material collected in the region of the University of Chicago. It was found that 1% peptone-glucose also supports sterile cultures, while yeast extract which has been passed through a SEITZ bacteriological filter seems to be equal to the standard medium for *Colpidium*. This is in agreement with BUTTERFIELD. It is in contrast to the findings of PETERS (1929), in which he states: "... I have been unable to make continuous sub-cultures of the strain used in the present experiments in the absence of solid matter in the medium".

The yeast extract seems to be eminently favourable to these *Colpidia*. Not only do they increase to great numbers, even as high as 200,000. per cc. (MR. BOND), but their appearance is uniform and excellent. They are fatter than their typical condition in nature, always showing numerous food-vacuoles which are clear. Presumably they ingest the medium. *Glaucoma pyriformis*, a facultative parasite, absorbs its medium to a large extent (LWOFF).

Method of sterilization by washing. Of the various methods of sterilization, washing in successive volumes of sterile water seems the most flexible and successful. The method of geotaxis urged by GLASER and CORIA (1930 p. 788) has been tried on two different occasions without success (in passing: *Polytoma* is not a ciliate (!). Some of the methods which have been used are reviewed by LUCK (1931) and by OEHLER (1924). The method of sterilization

by wandering from infected areas to sterile regions on an agar plate as described by OELHER (1919 p. 19) is, at best, limited to very small organisms.

The technic of sterilization by transfer through successive sterile baths has been improved by PARPART (1928). The method whose description follows is the result of extensive experience with a variety of Protozoa. The major innovation consists in the combination of migration ("Wandermethode" — BĚLAŘ) with washing ("Verdrängungsmethode" — BĚLAŘ).

Material. It is important to have the use of a room in which the air is quit. Under these conditions the hood described by PARPART is quite unnecessary. The temperature for all manipulations is preferably 20° or below, and the organisms undergoing sterilization were at no time incubated. A binocular microscope having a selection of low power objectives and equipped with side rests for the hand is preferable. The dishes which have proved the most generally useful are the isolation-dishes (hollow-ground squares of plate glass) previously mentioned, p. 258. They are to be placed in petri dishes, stacked, wrapped in paper, sterilized by hot air, and kept covered until used.

The most satisfactory pipettes are ordinary medicine droppers (Pipette mit Gummihütchen) drawn out to a bore suited to the size of the object to be transferred. The bulb may be removed, a plug of cotton inserted, and the whole sterilized before use, but in my experience this does not help. The whole pipette is to be heated very hot when drawn out, and should be flamed frequently during use. The sterile medium into which the organisms are to be finally inoculated should be used for the washing medium. A liberal supply made up in tubes should be on hand. The organisms which are to be subjected to this, at best, wearing process, should be vigorous and normal, and available in concentrated numbers in a medium which is isotonic with the final medium. They may be concentrated by centrifuging gently.

Method. The petri dishes enclosing the isolation dishes are arranged serially on a board so that they may be easily picked up for removal to the stage of the binocular. The pipettes are similarly arranged serially at the edge of the table so that their ends touch nothing. From 1 to .5 cc. of medium may now be introduced, from the side, into the first three or four isolation dishes. The

remaining dishes are filled as needed. The first petri dish is now placed on the stage of the microscope; the cover may be removed. One or two drops of concentrated organisms are carefully introduced at the left edge of the medium in the isolation dish. If this is performed properly the bacteria will be strictly circumscribed in a zone on the left of the dish. The Protozoa will move more rapidly than the bacteria, some of them reaching the opposite edge (right) in a brief time. While their movement is being followed under the binocular, a clean pipette is flamed. When the tip has cooled the Protozoa on the right may be removed and introduced in a similar fashion at the left edge of the second dish.

Having traversed about a centimeter of sterile medium, the organisms would now be sterile if it were not for two sources of difficulty. First, bacteria may adhere to the surfaces of the organism. Second, spores which have remained undigested will be defecated at a later time, as shown by PARPART (1928). Both difficulties are greatly reduced by using reasonably large, non-spore-forming bacteria such as *Pseud. fluorescens*, *Bact. coli*, *A. aerogenes* etc. If this is impracticable, it is necessary to delay transfer following the fifth washing for a period of 5 hours. Following this interval the organisms must be washed at least four more times. After the third washing the importance of migration across the field decreases, and the importance of keeping the covers over the petri dishes increases. Observation should be carried out through the petri dish cover. The organisms should be allowed to swim around for considerable periods between the last transfers, because adhering bacteria are thus thrown off. This technic is imperfect in this regard, for in the presence of certain bacteria of an unknown nature, it is impossible to obtain sterile ciliates. For instance, a repeatedly subcultured .1 % malted milk culture of *C. colpoda* yields ciliates which cannot be sterilized by this method. *C. colpoda* growing on *Pseud. fluorescens* may be sterilized in five washings. I have found an interval of one-half hour to one hour for the last two washings before the final one suitable intervals for delay to permit this sloughing off of adhering bacteria. The absolute number of times transfer is required for sterility depends on the size of the organism. *Paramecium*, for instance, may require as many as ten. When several or many sterile organisms are required, it is best not to carry more than 5 to 7 in a given series of transfers, but to begin again by introduction of a new concentrated mass in the second or third dish, and to add two fresh dishes at the end of the series.

Glaucoma scintillans.

Glaucoma scintillans is a very common ciliate and appears, like *C. campylum*, in the early stages of a hay infusion. It was found that addition of raw beef to cultures caused a marked increase in numbers.

No preliminary observations on culture on bacteria, etc., were made. Using the technique outlined for *C. campylum* in the last section, sterile specimens were placed in yeast extract.

Growth was very slow and large numbers were never obtained, yet the individual organisms appeared in good condition. Possibly the slow growth was referable to the mouth structures which may not permit ingestion of fluid in the absence of large numbers of particles in the medium. *Glaucoma* is apparently adjusted in the natural environment to great concentrations of bacteria, judging from the excellent growth obtained on raw beef infusions. If this is true, then the addition of sterile powdered egg, meat, malted milk, dead bacteria etc., might give interesting results. The recent development of "colloid mills" which permit the reduction of insoluble substances to particle sizes suitable for ingestion by ciliates opens up an interesting field in the sterile culture of Protozoa. It is also possible that the yeast extract was chemically deficient and could be improved upon by suitable additions.

Loxocephalus granulosus.

This organism resembles exactly the illustration given in CALKINS (1926, p. 383).

Using the technique outlined above, sterile culture was achieved in yeast extract and in peptone-glucose. Growth was comparable to that of *C. campylum*. This was announced in my paper on *Stentor* (1932).

Colpidium colpoda.

Sources. This ciliate, as BRESSLAU (1923) indicates, is much more rare than *C. campylum*. I have found it only twice, first in a polluted stream below Valpariso University (Indiana), and later in "Mud Lake", in the vicinity of Stanford University [1]). In each case it was associated with a varied fauna of large Protozoa, including *Stentor*, *Loxodes* and *Blepharisma*.

[1]) Collected by Prof. C. V. TAYLOR. The culture as brought in from the field contained manure in addition to decaying vegetation.

If specifically inoculated, this organism appears in large numbers after about two weeks in the typical hay infusion, and disappears after another week but may under some conditions persist considerably longer. In view of the ease with which it may be cultivated, it seems strange that it is not as ubiquitous as, for instance, *Paramecium.*

Inorganic medium. While it was soon found that *Colpidium colpoda* will slowly increase if placed in an infusion of wheat grains in tap-water, nearly all experiments have been carried out using a presumably very favourable salt medium, PETERS' medium, made up in double distilled water.

The cation ratio of this medium, and its possible general significance has been given in my paper on the culture of *Stentor* (1932). My opinion to the effect that it is is essentially a more favourable medium than the various modifications of the classical balanced physiological medium has been beautifully verified for *Amoeba proteus* in the recent paper by HAHNERT (1932). The anions have been modified for general use with two ends in view. First, the p_H has been reduced from 8 to approximately 7.5. Second, sulphate and phosphate anions have been added in order that it may serve as a basic inorganic medium to which organic substances may be added for the growth of bacteria. PETERS' medium follows:

$Ca(HCO_3)_2$	.00055 M.	.0059 %
$MgSO_4$	.00015 „	.0037 „
K_2HPO_4	.00015 „	.0026 „
Na_2HPO_4	.00015 „	.0027 „

With regard to preparation, it has been found from experience that the addition of excess CO_2 rather than a measured amount to achieve the desired p_H, not only expedites preparation but preserves the medium against the growth of bacteria and small monads which otherwise occurs. The volumes required for daily use are to be drawn off and shaken in a large beaker until in equilibrium with air, before use. For details of preparation see my paper. The inorganic components of a standard bacteriological medium are given here for comparison:

H_2O	.1 l	
$MgSO_4$	.2 g	.02 %
$CaCl_2$	.1 „	.01 „
K_2HPO_4	1. „	.1 „

Thus PETERS' medium is much more dilute, but the concentration of nutritive substances to be added, for my purposes, is also relatively

dilute. The concentration of $SO_4^{=}$ and $HPO_4^{=}$ in PETERS' medium is, as will be shown below, adequate.

Enrichment. The rather inadequate method of trial of substances for the growth of *Colpidium* was first used. Small amounts of the substances were placed in 100 cc. of medium in covered dishes which allowed a large surface for contact with air. Inoculation consisted of several *Colpidia* for each dish. In spite of its crudity and the totally unknown bacterial flora concerned, the method gave results, and the following list of substances tried is not without significance.

Substance	Growth
Wheat grains	large (slow increase)
Lettuce (not heated)	slight
Ground lettuce (not heated)	slight
Spinach „ „	slight
Ground spinach „ „	slight
Beef extract	slight
Hay (TIMOTHY)	fair
„Käseröhrchen" (PRINGSHEIM)	slight
Peptone .1 %	large (unstable)
Malted milk .05—.1 %	very large (rapid increase)

The malted milk was added to the dish as the dry powder just before inoculation. A quite dilute suspension is best to start with. If added to the original pond water containing the *Colpidia* increase is poor or nil. This is probably due to the large initial bacterial content. *C. colpoda* has been cultivated continuously for over a year in this manner, being used as a source of food for *Stentor*. When clean covered dishes of about 6 cm. diameter and 4 cm. depth are used (50 cc medium, add 50 mg. malted milk as dry powder), the danger of growth of lactic acid bacteria, which are very injurious, is negligible. It may apparently be said that under all other conditions *C. colpoda* will grow fast and normally for a few days, the organisms becoming quite opaque from the ingestion of malted milk particles. Transfer is accomplished by gentle centrifuging in 15 cc. tubes, a pipette being used to withdraw the concentrated mass. If transferred (centrifuge and add concentrated mass) during the period of rapid increase, which usually endures for three or four days, this increase will continue for many sub-cultures, but this is unsatisfactory over long periods because unfavourable bacteria apparently gain the ascendency. To avoid this it is simply necessary to wash (centrifuge) the organisms at intervals.

At a later time some additional substances were tried. They will be added here to complete the list.

Substance	Result
Asparagin .01—.1 %	fair
Casein (granular)	nil
Casein (powdered)	fair
Hemoglobin	nil
Cracked wheat	fair
Barley grains	slight
Macerated brain tissue	fair
Milk (dilute)	fair-large (unstable)
Aszitesflüssigkeit	large
PETTIJOHN's (rolled wheat)	large
Bran	slight
Wheat shorts	slight
Starch (several kinds)	nil
Germea	large
Sludge	slight
Powdered milk	large

Observation under the microscope showed that the particle-size, in the case of the powdered casein and the powdered milk, was too great for ingestion by *C. colpoda*. Examination of the malted milk suspension revealed, on the other hand, large numbers of particles which showed more or less Brownian movement, and which could be readily ingested by this organism. It thus becomes evident that the special virtue of malted milk lies in the fact that it is eaten, rather than in the enrichment of any particularly favourable bacterium.

Growth in suspensions of bacteria. The following bacteria were tried in suspension as for *C. campylum*. Growth was obtained in several cases, but in no case could it be maintained in sub-cultures.

Bacterium	Growth
yellow	fair
gray	nil
ochre	slight
mixture of above three	slight
Bacillus subtilis	large
Pseudomonas fluorescens	large
Bacterium coli	nil (toxic)
Proteus vulgaris	large
Aerobacter aerogenes	very large
Alkaligenes fecalis	fair
Aerobacter cloacae	fair
Micrococcus ureae	nil (innocuous)
Pseudomonas pyocyaneus	fair

Bacterium	Growth
Mycobacterium salmonicolor	slight
Aer. aerogenes + *Bact. subtilis*	large
Aer. aerogenes + *Ps. fluorescens*	fair
Saccharomyces cerevisiae (yeast)	nil

The growth indicated is for organisms transferred from a favourable medium (malted milk). Of those systems in which growth was obtained, sub-culture failed with one exception. In the case of *A. aerogenes* growth continued until the second transfer, when little or no further increase can be obtained. This result was verified by performing the experiment as it should be done; that is, using suspensions of carefully washed bacteria. *A. aerogenes* was grown on nutritive agar surfaces in large qantities and removed by means of a fine jet of water to 15 cc. centrifuge tubes, where it was washed three times. Two series of vessels were made up with a graded concentration of the washed *A. aerogenes* in suspension: 1. a series of 16 (each concentration in duplicate) isolation dishes containing 1 cc. of medium, 2. a series of 8 culture tubes containing 10 cc. of medium. *Colpidia* which had grown well through the first sub-culture were inoculated. Growth was slight or nil in the entire series.

All but the most dilute suspensions of *B. coli* killed *C. colpoda.* In sub-lethal concentrations the ciliate tends to grow, apparently in spite of the toxic influence.

Micrococcus ureae, in contrast to those bacteria on which growth was obtained, behaved as perfectly inert particles through which, in any concentration, the *Colpidia* plough with enthusiasm, but without the slightest result. The same is true of the only yeast tried, *Saccharomyces cerevisiae.*

To summarize, no bacterium was discovered which, when suspended in fresh water (PETERS' medium), would support growth of *C. colpoda* for more than a few generations.

Zweigliedrige Kultur[1]) in wheat infusions. In addition to the suspensions, the first six bacteria listed were tried on wheat infusions. 300 cc. of medium and one gram of wheat in each

[1]) BĚLAŘ uses the following terms to describe the degree of purity of cultures: Rohkultur: apparently = our "mass culture". Unreine Spezies Reinkultur = the culture object on a varied flora including food organisms. Eingliedrige Kultur = pure culture. Zweigliedrige Kultur = 2 organisms in pure culture, one serving as food for the other. Dreigliedrige Kultur = 3 organisms in pure culture, serving progressively as food. The last two terms are useful, and will be used in my further work.

of six cotton stoppered bottles were autoclaved and inoculated with 5 *Colpidia* each. These *Colpidia* had been washed through five sterile transfers according to the method outlined above. They were not checked for sterility. Results are given in the following table (figures represent p_H):

Bottle No.	Inoculum	Growth in: 10 days		20 days		90 days	
1	yellow	large	7.3	small	7.	nil	
2	gray	very large	7.	very large	7.3	nil	
3	ochre	large	7.	large	7.	nil	
4	*Bac. subtilis*	large	7.	very large	6.8	nil	
5	*Ps. fluorescens*	large	7.3	large	7.	nil	
6	*Bact. coli*	nil	7.4	nil	7.6	very large	7.6

The p_H changes show no correlation with degree of growth. In the case of *Bact. coli* a fine growth of *Colpidium* followed three months of activity on the part of this bacterium. The cotton plugs in the bottles were covered with tinfoil to reduce the possibility of contamination over the long period, but it is possible that another organism gradually gained the ascendency since the *Colpidia* inoculated were not certainly sterile. No explanation is offered for this behaviour in the case of *Bact. coli*, but it is an interesting fact in the light of the relations to the hay infusion cycle, to be considered in the discussion. Zweigliedrige Kultur with *Pseud. fluorescens* is considered below.

Test of possible conditioning effect of malted milk. The rapid increase which characterizes cultivation in malted milk has been maintained through innumerable transfers for more than a year. The introduction of *Pseud. fluorescens* or *Bact. subtilis* at the beginning of one of these sub-cultures has no effect on the result unless added in quantities sufficient to make a slightly cloudy suspension, in which case normal increase is retarded. Meanwhile it had been found that the ciliate will not grow in suspensions of sterile malted milk (see p. 267). Accordingly an experiment was arranged to test the conditioning effect of malted milk by the growth of *C. colpoda* on *Pseud. fluorescens* in the conditioned system through the addition of more bacteria at later intervals.

Colpidia were started in the usual way. It was found that after 24 hours about 5 % were visibly dividing when observed at a magnification of 20×. Additional *Pseud. fluorescens* was added in a graded series, and a control in which no bacteria were added was run simultaneously. The next day (= 2 days) about 7 % were

dividing in the control while the series to which additional fluorescens had been added ranged from 5 % in the most dilute suspension of bacteria to 2 % in the most concentrated. On the third day control organisms were still increasing, opaque, and well nourished in appearance, while experimental organisms became transparent. The dishes were discarded on the twenty-fifth day, when they were in the following condition. Controls, transparent but normal in appearance and strongly aggregated on one side of vessel; more concentrated experimentals, dead, less concentrated, reduced in number and size and radically vacuolated. This aggregation is characteristic of the normal malted milk culture, commencing at about the time increase ceases.

Thus the presence of large numbers of *Pseud. fluorescens* proved injurious to *Colpidium*, yet the presence of some bacteria, at least, seemed to be necessary. We may again recall CHATTON'S 1927 paper describing the cytolytic action of chromogenic bacteria. *Pseud. fluorescens* was one of the three bacteria found to be most active in causing cytolysis of *Glaucoma scintillans.*

Zweigliedrige Kultur on *Micrococcus ureae.* Assuming that the active growth of limited numbers of bacteria was the crucial requirement met by the malted milk, a simple known system involving the growth of *Micrococcus ureae* on glucose was tried. It was known that suspensions of this organism in water were utterly innocuous both with regard to toxicity and to nutritive adequacy for *Colpidium*, hence positive results in a nutritive system would be immediately significant. Sixteen tubes of different proportions and concentrations of glucose and urea were made up and inoculated, together with controls consisting of a series of suspensions of *Micro coccus* in the inorganic medium (PETERS' medium). Results were uniformly negative, although the individual *Colpidia* inoculated continued to swim about for at least a week.

Zweigliedrige Kultur on *Pseudomonas fluorescens.* This bacterium growing on the amino-acid asparagin would constitute a good culture method because: 1. being the enrichment culture method for *Pseud. fluorescens* it should be stable in the absence of rigidly sterile methods, 2. the metabolites are known, being CO_2, NH_3, and H_2O.

Culture tubes containing .5 %, .1 %, .05 %, .025 %, .01 %, and .005 % asparagin in PETERS' medium (10 cc.) inoculated with *Colpidia* and *Pseud. fluorescens* were made up. Overgrowth of the bacteria killed all but the .01 % and .005 % systems, in which growth was

good. Sub-culture (.01 %) failed after the second transfer: there was no appreciable growth following transfer to the third tube which occurred on the fifteenth day of Zweigliedrige Kultur. The bacteria increased uniformly.

Apparently indefinitly continued culture may be achieved using wheat (3 grains in 50 cc. PETERS' medium autoclaved in 125 cc. ERLENMEYER flasks). Sub-cultures were also made on dilute peptone glucose, yeast extract, and sterile .1 % malted milk with excellent growth. Although sterile transfer may usually be achieved by pouring from one vessel to another using the customary flame technique, this is not to be depended on for routine use. Pasteur pipettes are to be used for transfer with stock vessels, sub-cultures from which may be manipulated as desired. This pseudomonad, as OEHLER (1924, p. 118) indicates, overgrows all other bacteria, and is in this regard rather easy to handle.

Zweigliedrige Kultur on *Proteus vulgaris.* Growth on wheat, peptone-glucose, yeast extract, and sterile .1 % malted milk is entirely comparable to the behaviour on *Pseud. fluorescens.* JOLLOS (1921, p. 8) states that *Proteus vulgaris* ("*Bacterium proteus*"), in his experience, overgrows most other bacteria. It may others, but it certainly does not compete with *Pseud. fluorescens.*

Zweigliedrige Kultur on *Aerobacter aerogenes.* My first isolations, all of which were made in autoclaved wheat, failed because this bacterium will not grow on wheat. The second effort was on sterile milk diluted 1:50 with double distilled water. Six to seven days were required for the protozoa to reach great numbers and overcome the cloudiness due to the growth of the bacteria and to the milk itself. At the end of about three weeks the Protozoa begin to disappear (20° C). When 1:100—1:500 milk is used (vol. — 40—50 cc.) the *Colpidia* increase for a period well over a month and have an excellent appearance but do not reach the great numbers characteristic of the 1:50 dilution. Sub-culture behaves as described for the initial isolation if the inoculum is small. If the inoculum is equal to $^1/_4$ of the total sub-culture volume no overgrowth of bacteria is appreciable, the ciliates increase to great numbers, and appear macroscopically to increase in a uniform manner quite as in the case of *C. campylum* in pure culture. Growth on .1 % peptone-glucose made up in PETERS' medium is roughly equivalent to 1:50 milk, and is excellent.

Still another effort to grow *C. colpoda* on bacteria nourished by relatively simple substances was attempted. KOSER's uric acid-

glycerol medium for *A. aerogenes* was chosen, these substances being simply added to PETERS' medium. Keeping the .05 % uric acid concentration constant, for it is a rather insoluble substance, the following concentrations of glycerol were made up: 0.1 %, 0.3 %, 0.5 % . %. Using small inocula of bacteria and ciliates, growth of *Colpidia* was nil in the .1 % concentrations, and practically so in most of the others. In four of the .3 % and .5 % tubes however, fair growth was obtained. Some of the organisms in these tubes showed very plain signs of injury (dwarfed size), while others were extremely healthy and active. Was this possibly the consequence of anaerobic metabolism in the lower part of the tube? This growth required ten days, and appeared still better after twenty days. Even less promising was growth in a corresponding series carried out in 125 cc. ERLENMEYER flasks, where the medium, in volumes of 40 cc., was relatively shallow. They had been given up as failures but had not been discarded after a period of three weeks, when an inspection showed very good growth in all of the .3, .5, and . % flasks. Growth was still better after four weeks. Sub-culture proved impossible. All of these cultures were strictly zweigliedrig.

In view of the suspension-experiments, of these results, and of those on asparagin, we may make the tentative assumption that *Aer. aerogenes* is inadequate as a sole source of nutrient for *C. colpoda.*

Aer. aerogenes shows no capacity to compete with other bacteria, and in my experience, contaminants promptly become evident. It is of course necessary to obtain sterile ciliates in order to establish a Zweigliedrige Kultur, and its maintenance, once established, is perhaps slightly more difficult than that of a pure culture in liquid media. Nevertheless, if the investigator is perfectly familiar with elementary bacteriological technic, the maintenance of such cultures is a simple routine procedure.

Attempts to achieve sterile culture. A very extensive series of experiments have been devoted to the sterile culture of this ciliate. Failure has resulted in all cases. The methods are here summarized.

1. Peptone-glucose. The *Colpidia* will tolerate .5 % of each and swim about for at least a week, but there are no signs of growth.

2. Yeast extract. An extensive series of tubes were inoculated on two different occasions. The *Colpidia* respond as for peptone-glucose. The addition of dead bacteria does not help, but may be injurious if the concentration is too great.

3. Malted milk. Autoclaved, fractionally sterilized (Tyndallyzed), filtered (Chamberland filter delivering a clear and sterile solution), and malted milk sterilized by ultra-violet light failed. Autoclaving and fractional sterilization caused coagulation of some of the components. Filtration delivered a particle-free solution, while the solutions sterilized by ultra-violet light, although resembling exactly the fresh suspension of malted milk, were toxic to the organisms. Thus the possibility still remains that if a non-toxic sample of the latter system could be obtained positive result migth be achieved.

4. Milk. The excellent appearance and rapid growth of *Colpidia* sealed between a slide and cover-slip in 1 : 50 "grade A" milk suggested the possibility of sterile culture on milk. Concentrations of 1 : 10 and 1 : 50 of sterile milk were tried with and without the addition of *Aer. aerogenes* killed by heating to 70° C. The *Colpidia* lived for as many as eight days, swimming about slowly, but no increase was ever obtained. The sterile milk had been centrifuged and contained very few fat droplets. The ciliates in the grade A milk ate the smaller fat droplets very actively.

Discussion.

Interpretations from the Hay Infusion: *Colpidium campylum* and *Glaucoma* appear early in a typical hay infusion which has been inoculated at the start with pond water. The period of their increase coincides with the time of increasing acidity in the infusion. Although *Colpidium colpoda* will not appear, in my experience, unless specifically inoculated, its period of increase occupies a well defined phase of the infusion-cycle: that which is characterised by a decreasing acidity on the acid side of neutrality. Of a number of records kept of the hydrogen-ion concentration changes in Timothy hay infusions, a fairly typical one [1]) (vol. = 4 L) is here rendered (Fig. 1 p. 275) together with a portrayal of the sectors occupied by the ciliates, because in it the time of their appearance and disappearance was accurately recorded. *Stentor coeruleus* has been included to complete the picture, for this heterotrich increases in the alkaline phase of the cycle until it is "decayed out" and ready to support autotrophic Protozoa. In this particular hay infusion *Loxocephalus* appeared on the 19th day, one day before *Stentor*, but it has not been observed frequently enough to justify any description of its characteristics.

[1]) It is interesting to compare this curve with that of JONES (1930).

Colpidium campylum grows in that period of the hay infusion which is characterised as follows:

1. The bacteria are decomposing a variety of relatively readily available carbon compounds which are complex.

2. The bacteria enriched grow very rapidly, tending to make the medium cloudy due to their great numbers.

3. The metabolites appear in relatively high concentration, are often acids, and are relatively toxic to other living organisms.

4. It is an environment which under natural conditions is typically transient.

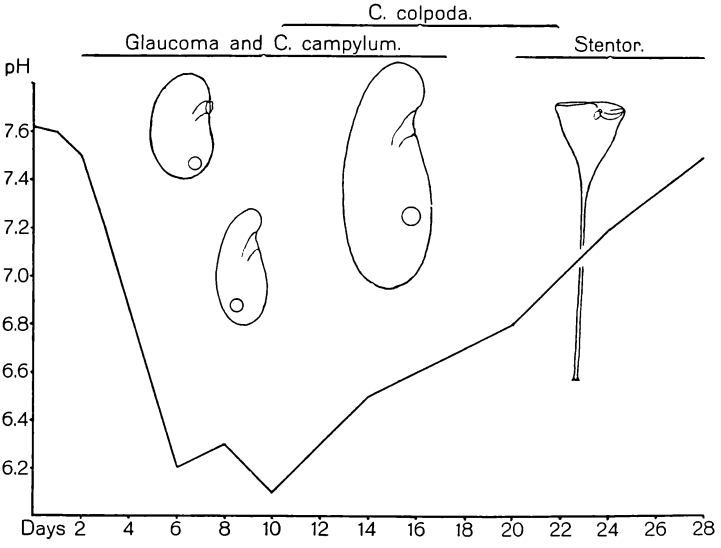

Fig. 1. Relations to the Hay Infusion cycle. (*Stentor* is relatidely twenty times reduced.)

Some characteristics of *Colpidium campylum* may be correlated as follows:

1. It is a relatively small and remarkably resistant organism: it will tolerate .3 % artificial sea water, 33 % yeast autolysate, a suspension of *Bact. coli*, and systems of growing *Bact. coli*.

2. It will tolerate thick suspensions of bacteria, including *Bact. coli*. (It is nevertheless possible to injure them by very thick suspensions of the latter.)

3. It will tolerate, in contrast to *C. colpoda* and *Stentor*, the metabolites of *Bact. coli*.

4. When placed in fresh water in the absence of a food supply it will, in contrast to *Stentor*, encyst.

Glaucoma manifests similar characteristics. A correlative investigation should form an interesting study.

Colpidium colpoda grows in that period of the hay infusion which is characterised as follows:

1. The bacteria of the first phase have consumed the soluble complex substances providing the requirements for very rapid growth. The bacteria are now constrained to utilize the decomposition products formed by preceding life and other less available infusion substances.

2. The bacteria enriched are different from those of the first phase and accumulate to great numbers causing a cloudy system.

3. The metabolites, at least from the standpoint of the Protozoa, are less toxic.

4. It is an environment which may be more persistent under natural conditions.

Some characteristics of the organism may be correlated as follows:

1. *Colpidium colpoda* is twice as large as *C. campylum*; it is distinctly less resistant. It will not tolerate a suspension of *Bact. coli* unless very dilute.

2. It will grow in infusions which do not suffer a marked initial p_H change even when very cloudy; otherwise growth is delayed.

3. It will not tolerate the metabolites of *Bact. coli.*

4. *C. colpoda* is more persistent in fresh water than *C. campylum.* In the absence of a food supply it will live for long periods. I have never seen it conjugate or encyst. The general fact that suspensions of bacteria will not support growth while the same bacteria growing in nutritive systems are favourable for continued increase, is a striking and interesting characteristic which certainly deserves further study.

The heterotrich *Stentor coeruleus* grows in that period of the hay infusion which is characterized as follows:

1. The bacteria are confined to the simple decomposition products now present in the medium, and to relatively insoluble substances such as cellulose, which are decomposed by a specialized microbial flora.

2. The bacteria enriched are accordingly limited in their rate of growth, and also, with regard to the solid bodies representing the insoluble substances, in their position in the medium, for they aggregate at the organic surfaces there. The medium thus clears

because of this limited growth, of the subsidence [1]) of the previous types of bacteria, and of their consumption by Protozoa.

3. The concentration and toxicity of metabolites are greatly reduced.

4. It is an environment which may be conceived to be quite lasting under natural conditions, both in polluted slow-moving streams and permanent bodies of fresh water.

Some characteristics of the organism may be correlated as follows:

1. *Stentor coeruleus* is one of the largest of the fresh water Protozoa and is promptly killed by .06 % sea water, yeast extract (10 % yeast autolysate), peptone-glucose, suspensions of *Bact. coli* and *Pseud. fluorescens* and all media made up in ordinary distilled water.

2. It will not tolerate suspensions of *Bact. coli* or of *Pseud. fluorescens.*

3. It will tolerate the dilute metabolites of certain growing bacteria, not including *Bact. coli* and *Pseud. fluorescens.*

4. It is remarkably persistent in fresh water, and will not conjugate or encyst in the absence of a food supply. It will conjugate and encyst under particular conditions of bacterial increase.

I do not offer the above generalizations as a complete statement of the ecology of the organisms studied. It is hoped merely that some of the correlations may prove fruitful.

It is unfortunate that the numerous investigations of the hay infusion do not provide data for a more specific analysis than the above. Hargitt and Fray (1917) consider some of the bacteria with reference to *Paramaecium.* They did not try suspensions of *Bact. subtilis* in fresh water as a source of food, comparing growth on growing bacteria.

It is apparent from the foregoing analysis that the hay infusion may be used as a tool in investigating Protozoa.

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[1]) I offer as a tentative suggestion that the Hypotricha enter into the picture here, as feeders on the bacteria and other bodies which have undergone subsidence, or on other aggregations of bacteria at surfaces. Thus Luck, Sheets, Thomas (1931) have been able to grow *Euplotes Taylori* on suspensions of *Bact. coli* + *Pseud. fluorescens* for a little over three years. Johnson (1932) obtained excellent growth of his *Stylonychia* on suspensions of *Pseud. fluorescens.*

It is also apparent from a review of the literature that inocula of hay, leaves, water plants, or earth, do not bring about growth of all the Protozoa which will grow at some stage of the infusion.

If a uniform series of infusions (vol. 4 l. or more) which would drop to a p_H of 6 when inoculated, for example, with 1 gram of garden earth, were inoculated with 5 cc. of pond water supporting large numbers of a specific organism, and if this were repeated for a number of organisms, comparative measurements of predominant bacteria, protozoan sequence, p_H, oxygen tension, etc., could be made which would prove fruitful. The relation of oxygen tension to the growth of such forms as *Urocentrum turbo*, *Metopus*, *Caenomorpha*, *Ludio*, etc., should prove interesting.

Comment on the life cycle. From the foregoing, studies on the "life cycle" of the ciliates considered might be predicted to give the following results: *C. campylum*, immortal; *C. colpoda*, few generations if cultivated on suspensions of bacteria, large number of generations if cultured on malted milk, immortal if maintained in zweigliedrige Kultur on milk + *A. aerogenes*; *Stentor*, two to four generations if placed in fresh water + unknown bacteria, many generations if cultivated in presence of certain bacteria and a suitable food organism.

I am indebted to KARL BĚLAŘ for an introduction to culture methods, to C. B. VAN NIEL for help in the culture of *C. campylum*, to P. J. BEARD for help in the culture of *C. colpoda*, and to C. V. TAYLOR for advice and assistance throughout the work.

Summary.

1. *Colpidium campylum* will grow on suspensions of a number of bacteria tried. It may be enriched and grown on suspensions of *Bact. coli.*

2. *C. campylum* may be grown in pure culture on particle-free yeast extract or peptone-glucose. It was concluded that it will not grow on inorganic sources of nitrogen; that its nutritive requirements resemble those of *Glaucoma piriformis.*

3. *Colpidium colpoda* will not grow on suspensions of a number of bacteria tried.

4. *C. colpoda* may be enriched and grown on malted milk in the presence of bacteria.

5. It will grow in Zweigliedrige Kultur on *Pseud. fluorescens*, *Proteus vulgaris*, and *Aer. aerogenes* in complex nutritive systems such as dilute peptone-glucose. It will not grow on *Pseud. fluorescens* on asparagin, nor on *Aer. aerogenes* on uric acid-glycerol.

6. The results of a long-continued series of experiments to achieve sterile culture were negative.

7. *Glaucoma scintillans* will grow, but only rather poorly, on yeast extract in pure culture.

8. *Loxocephalus granulosus* will grow in pure culture on yeast extract and peptone-glucose.

9. Some relations to the hay infusion cycle are discussed.

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