(Biological Laboratory, University College, New York University.)

Growth of *Glaucoma ficaria* Kahl in cultures with single species of other microorganisms.

By

David F. Johnson.

(With 1 figure in the text.)

The literature contains much information on cultures of ciliates with other microorganisms, and such investigations have been reported frequently in the past few years. Probably one of the first attempts to restrict the diet of ciliates was that of Powers (Powers and Mitchell, 1910) who succeeded in growing *Paramecium caudatum* and *P. multimicronucleata* first on *Chilomonas* and later on small ciliates, although no attempt seems to have been made to exclude all bacteria. Powers stated that *Paramecium* is almost exclusively a bacteria-feeder and that these changes in the diet induced abnormalities, both nuclear and cytoplasmic, in the ciliates.

Hargitt and Fray (1917) were apparently the first to grow ciliates on pure lines of bacteria. Growth of *Paramecium caudatum* and *P. aurelia* in pure cultures of bacteria isolated from infusions was compared with that in mixed cultures of bacteria. In such cultures, *B. subtilis* was the only species found to support satisfactory growth of *Paramecium*. These workers concluded, therefore, that a mixture of bacteria seemed essential in the diet of *Paramecium*. This general conclusion was supported by Phillips (1922), who found that pure cultures of *Bact. coli*, *B. cereus*, *B. proteus* and nine unidentified species would not support good growth of *Paramecium aurelia*. Fair growth was obtained in pure cultures of an unidentified *Streptothrix*, but the results were much less satisfactory than in her

mixed cultures of bacteria. PRINGSHEIM (1928) found that *Azotobacter* and *B. flourescens* would not support good growth of colorless *Paramecium bursaria; B. proteus* was satisfactory for a time, but later proved inadequate. LIKEWISE, *Azotobacter*, was found to be unsuitable for *P. caudatum*. GLASER and CORIA (1930) experienced similar difficulties with *Paramecium caudatum*, being able to grow the ciliate only on one unidentified species of bacteria. RAFFEL (1930) likewise failed to grow *P. aurelia* on pure cultures of bacteria, although good results were obtained with a mixture of *B. candicans* and an alga, *Strichococcus bacillaris*. In the case of *Euplotes taylori*, LUCK, SHEETS and THOMAS (1931) found that on pure strains of *B. coli communis*, *Sarcina citrea*, *Rhodococcus roseus* and three unidentified species the ciliates grew poorly if at all, whereas good growth occurred in mixtures of *B. coli* and either of two of the unidentified species. Sterile filtrates of thriving bacterial cultures, bacterial dialysates, autolyzed bacteria, phage-lysed bacteria, toluene-killed bacteria, and heat-killed bacteria all failed to support growth of *Euplotes taylori*. It was discovered later that, under certain specified conditions, one of the three unidentified species of bacteria (probably *B. flourescens pseudomonas*) would support growth of the ciliate.

Other workers have been more successful in their attempts to grow ciliates in pure cultures with a single strain of bacteria. OEHLER (1919—1924), who has done extensive work in this field, found that *Colpidium campylum* thrived in pure cultures of *B. subtilis*, *B. prodigiosus*, *B. coli*, *B. fecalis alcaligenes*, *B. pyocyaneus*, *Spirillum volutans*, *Staphylococcus aureus*, *Sarcina lutea* and *Corynebacterium diphtheriae*, but failed to grow on dead bacteria. *Colpoda steinii* and *C. cucullus* were cultivated in cultures with several different species of bacteria, and *C. steinii* also grew on heat-killed *B. coli*. *Paramecium aurelia*, *P. caudatum* and *P. putrinum* grew well in cultures of *B. fluorescens*, contrary to the earlier findings of HARGITT and FRAY (1917). OEHLER pointed out that gram-negative species serve better as food for ciliates than gram-positive ones, and that acid-fast species cannot be digested by many ciliates. AMSTER (1922) grew *Balantophorus* on a pure line of a gram-negative bacillus, later identified by OEHLER (1924) as *B. fluorescens*.

Later workers seem to have experienced little difficulty in growing ciliates on a number of species of bacteria in pure cultures. *Glaucoma scintillans* was grown by E. and M. CHATTON (1923) on both *B. coli* and *B. fluorescens*, but not on *B. flavus*. *Colpidium* has been grown by CUTLER and BAL (1926) on *Azotobacter* by BUTTERFIELD

(1929), on *B. aerogenes'* and by HETHERINGTON (1933), on *B. subtilis*, *B. coli*, *B. prodigiosus*, *Ps. fluorescens*, *B. pyoseptica*, *B. kelinensis*, *B. phymonthensis*, and five unidentified species. Successful results have been obtained with *Paramecium aurelia* by ANDREJEW (1928), using pure cultures of *B. subtilis* and *B. megatherium;* and by PHELPS (1934), with *B. prodigiosus*. PHELPS stated, however, that the bacteria must be supplemented by additional particulate matter in the culture medium, and that bacteria killed by physical and chemical means were entirely unsuitable as food for *P. aurelia*. *Paramecium caudatum* has been grown by MICHELSOHN (1928) on a strain of *B. subtilis*; by LUDWIG (1928), on the same bacillus; and also by HETHERINGTON (1934 b) on *Achromobacter pinnatum* LOSINA-LOSINSKY (1931), in studying the feeding reactions of *Paramecium caudatum*, found that this ciliate reacts quite positively to suspensions of *B. subtilis*, less positively to *B. fluorescens*, and only slightly to *B. coli communis*. *Paramecium multimicronucleata* has been maintained by GEISE and TAYLOR (1935) on a strain of *Pseudomonas ovalis* for a period of 26 months. Five unidentified species were unsatisfactory for mass cultures of the ciliate, although two supported moderate growth in isolation cultures. According to BARKER and TAYLOR (1931), *Colpoda cucullus* grows well on pure cultures of *Pseudomonas flourescens*. JOHNSON (1933) has found that *Pseudomonas flourescens* and *B. subtilis* will support good growth of *Oxytricha fallax*, the former being the better. Six unidentified species failed, in pure culture, to maintain growth of the ciliate.

Microorganisms other than bacteria have been used as food for ciliates by several investigators. LUND (1918) found it possible to grow *Paramecium caudatum* in tapwater to which fresh yeast was added. She used isolation cultures but did not attempt to prevent contamination other than by daily washings and changes to fresh supensions of yeast. OEHLER (1924) reported good cultures of *Colpoda steinii* and *C. cucullus* on *Saccharomyces exigus*, and also with diatoms, other algae, and fungus spores. *Colpidium campylum*, however, failed to thrive on *Saccharomyces exigus*. PRINGSHEIM (1928) found that two species of yeast, *Saccharomyces exigus* and *Schizosacchromyces Pombe*, were especially suitable as food for *Paramecium bursaria*. *Chlorella*, *Prototheca* and *Zopfii*, two species of *Stichococcus*, and a species of *Hormidium* were almost as good as the yeast. The colorless *P. bursaria* was able to establish a sort of symbiotic association with *Stichococcus* and *Hormidium*. *Polytoma uvella* and *Chlorogonium elongatum* also supported very good growth of *P. bursaria*, while *Cos-*

marium and *Euglena* were less satisfactory. WEYER (1930) cultured *Gastrostyla steinii* first on *Chlorogonium elongatum* and later on *Gonium pectorale*. LOEFER (MSS) has observed excellent growth of *Paramecium caudatum* in pure culture with the yeast, *Saccharomyces cerevisiae*.

Toxic effects, or other unusual effects of bacteria on ciliates, have been reported by certain workers. PHILPOT (1928) found that, under certain conditions, cultures of *B. pyocyaneus* are toxic to *Paramecium aurelia*, *P. calkinsi* and *P. caudatum*, but that the ciliates develop a resistance to the toxic substances and subsequently grow well in cultures of this bacillus. On the other hand, *B. enteritidis* was lethal to *Paramecium*, which in this case was unable to develop a resistance. E. and M. CHATTON (1925—1929) found that pure lines of a number of species of bacteria have different effects on the occurrence of conjugation in *Glaucoma scintillans*, some species appearing to inhibit conjugation completely. They reported also a cytolytic action of certain bacteria on *Glaucoma*, *Colpidium*, *Colpoda* and *Paramecium*, with the most marked effects being produced by chromogenic strains of *B. flourescens* and *B. pyocyaneus*. It was found that growth of *Glaucoma scintillans* on dead *B. coli* and *B. flourescens* could be obtained if the cultures were started in a medium which had previously been acted upon by the bacteria. In other words, if the dead bacteria were removed from their own medium and transferred with the ciliates to fresh medium, no growth of the latter occurred. LUCK, SHEETS and THOMAS (1931) noted a definite toxic effect of an unidentified species of bacteria on *Euplotes taylori*.

The present investigation was undertaken to determine whether *Glaucoma ficaria* can live indefinitely with pure line cultures of microorganisms as the only source of food, and also to determine the effects of such diets on the metabolism of the ciliates. The microorganisms so investigated include bacteria, yeasts, mold spores, alga and flagellated protozoa. The writer wishes to express his appreciation to Prof. R. P. HALL for his suggestions and criticisms during the course of the investigation.

Material and methods.

The strain of *Glaucoma ficaria* used in the present investigation is the one previously isolated in bacteria-free cultures by the writer (JOHNSON, 1935/36). STOCK cultures of the ciliate have been maintained in peptone medium for 6—8 months. The various species of bacteria, yeasts and molds were obtained from the Parke Davis Co., Detroit,

Michigan. The *Chlorella*, *Chlorogonium* and *Chilomonas* cultures were supplied by Dr. J. B. LOEFER; and the writer is indebted to Prof. R. P. HALL for the cultures of the various *Euglena* and *Astasia*. The bacteria, yeasts, molds and the alga, *Chlorella*, were cultivated on agar slants, while the small flagellates were grown in dilute peptone solution.

In practically all of the previous investigations on ciliates in cultures with bacteria and other microorganisms, the workers have used media containing dissolved or suspended organic matter, which probably served as an additional source of food for the ciliates. Since the ciliate, *Glaucoma ficaria*, used in the present investigation is capable of continued existence in synthetic, bacteria-free media, it was necessary in determining the suitability of a particular food-organism to exclude all other nutritive material from the experimental cultures. This was facilitated by preparing suspensions of the food-organisms in a non-nutritive salt solution, by washing the ciliates free of nutritive material previous to inoculation of the experimental cultures, and by excluding contamination from outside sources.

PRINGSHEIM'S (1928, p. 310) salt solution was used in all experiments unless stated otherwise. The composition is as follows:

$Ca(NO_3)_2$.	0.2 gm.
$MgSO_4$.	0.02 gm.
K_2HPO_4	0.02 gm.
NaCl .	0.02 gm.
$FeSO_4$	trace
Distilled water. . . .	1.0 liter.

For experimental purposes the ciliates were grown in Pyrex culture tubes plugged with cotton. All experimental cultures were incubated at 28° C. in a water bath. The p_H of the media was adjusted by means of a LA MOTTE roulette comparator. A Sedgwick-Rafter counting chamber and a Whipple micrometer was employed in determining the initial and final counts of the ciliates.

STOCK cultures of the ciliates were grown in peptone medium. Since this medium, even in dilute solution, will support growth of the ciliates it was necessary to exclude all traces of it from the experimental cultures. This was accomplished by repeated washes in sterile salt solution. The ciliates were placed in a sterile, cotton-stoppered centrifuge tube and centrifuged. The supernatant liquid was drawn off with sterile pipettes and sterile salt solution

was added. This washing process was repeated four times and at the end of the fourth wash the ciliates were suspended in 100 cc. of sterile salt solution contained in an EHRLENMEYER flask. Such suspensions were prepared for each experiment to serve as the source of the inoculum. This same washing procedure was followed in preparing suspensions of the flagellates, which were grown in peptone media, and also of the washed bacteria when used. Otherwise, suspensions of the food-organisms, including bacteria, yeasts, mold spores and algae, were prepared by removing the organisms from agar slants with a sterile platinum loop and introducing them into sterile salt solution.

It would obviously be impossible to control exactly the number of individuals in preparing suspensions of the food-organisms. This difficulty was compensated for by preparing the different suspensions in approximately the same density, as judged by comparison with a standard. A dense suspension of heatkilled *B. lactis aerogenes* was used as the standard. The food-organisms were added in suspension until the density approximated that of the standard. Since an excess of food was provided in all experiments, as evidenced by the number of food-organisms remaining at the end of the experimental period, the approximation of equally dense suspensions of the food-organisms is probably justified.

LWOFF (1932) concluded that a medium which would maintain the growth of a protozoa through three successive transfers contained the substances essential for the continued growth of the organism. In the present investigation fifteen successive transfers, made at two day intervals, were used as a criterion in determining whether a certain species of microorganism in mixed suspension with *Glaucoma ficaria* is capable of supporting indefinite growth of the ciliate.

Bacteriological methods were strictly adhered to in all of the experimental work. All of the equipment used was sterilized in the usual manner, and transfers were made in a room in which there was minimal air disturbance. Agar plates were systematically inoculated to test the purity of the cultures.

With the procedure outlined above it was possible to control the food supply, having only the experimental food-organism available as food for *Glaucoma ficaria.* It was possible also, by making successive transfers over a period of from one to five months, to be reasonably certain that such cultures could be carried indefinitely.

Living bacteria as a source of food.

Series I.

Suspensions of seven non-pathogenic species of bacteria were prepared in sterile PRINGSHEIM's medium and then inoculated with washed *Glaucoma ficaria* as outlined above. PRINGSHEIM's medium alone was inoculated with the ciliates for use as a control. The initial count was adjusted to 500 organisms per cubic centimeter. The cultures were incubated for 48 hours, transfers were made to fresh suspensions of bacteria and the original cultures were then fixed for determination of the final concentration of ciliates. Growth is expressed in table 1 as x/x_0 (ratio between final and initial concentrations of organisms), and the results of subsequent transfers are listed.

Table 1.

Growth of *Glaucoma ficaria* in suspensions of non-pathogenic bacteria; x_0, 500 per cc.

Species	Initial pH	Final pH	x/x_0	Transfers 1—15	Transfers 16—30
B. subtilis	6.4	6.4	24.8	+	+
B. mucosum capsulatum	6.3	6.4	39.4	+	+
Bact. lactis aerogenes	6.9	7.2	68.0	+	+
Bact. aceti	6.5	6.6	37.3	+	+
Mycobacterium smegmatis	6.5	6.5	1.2	—	
Spirillum rubrum	6.4	6.5	15.8	+	+
Proteus vulgaris	6.8	7.0	23.9	+	+
Control	6.2	6.2	0.9	—	

Some growth of the ciliates occurred in all the experimental cultures except the control, the increase being the greatest in suspensions of *Bact. lactis aerogenes*, while growth on *Mycobacterium smegmatis* was negligible. Repeated transfers were successful with the exception of the control and the suspensions of *M. smegmatis*. In these latter two, the ciliates were all dead at the end of the fourth transfer. The fact that the ciliates survived as long in the *M. smegmatis* suspensions as they did in the control indicates that the failure of this bacterium to support growth of *Glaucoma ficaria* is probably not due to any toxic effect. The result can readily be attributed to the growth characteristics of this bacterium, since *M. smegmatis* forms dry conglomerate colonies on agar slants and when introduced into salt solution these aggregates of bacterial cells remain intact, thereby making ingestion by the ciliates impossible. Attempts to grow *G. ficaria* in suspensions of this

bacterium were repeated several times with negative results. Transfers of the successful cultures were made at two day intervals for a period of 30 days and thereafter sub-cultures were made once a week. *Glaucoma ficaria* was maintained for five months on suspensions of six of the seven species of bacteria investigated in this series. This would seem to indicate that these species are capable of supporting indefinite growth of *G. ficaria* in pure culture without any additional organic food.

Series II.

A similar procedure was followed in investigating the growth of *Glaucoma ficaria* on suspensions of ten species of pathogenic bacteria. As in the previous series PRINGSHEIM'S medium was used as a control. The initial count was 450 organisms per cc., and the cultures were incubated for 48 hours. The results of this series are given in table 2.

Table 2.

Growth of *G. ficaria* in suspensions of pathogenic bacteria: x_0, 450 per cc.

Species	Initial pH	Final pH	x/x_0	Transfers 1—15	Transfers 16—30
Bact. coli	6.7	6.7	35.3	+	+
Bact. typhosus	6.3	6.6	15.5	+	
Bact. paratyphosus A	6.4	6.6	21.3	+	+
Bact. paratyphosus B	6.3	6.9	24.7	+	+
Bact. enteritidis	6.5	6.6	21.6	+	+
Bact. pyocyaneus	6.8	7.2	42.9	+	+
Bact. dysenteriae Shiga	6.5	7.0	42.0	+	
Staphylococcus albus	6.3	6.5	9.0	+	+
Staph. aureus	6.2	6.3	22.0	+	+
Staph. citreus	6.5	6.7	14.9	+	+
Control	6.2	6.2	0.8	—	

Growth occurred in all the cultures in this series, and these cultures were successfully transferred for periods of from one to five months. Transfers were made as before, the first fifteen at two day intervals and subsequent ones at intervals of seven days. As in the previous series no growth occurred in the control. An interesting feature of this series was that maximal growth of the ciliates occurred in suspensions of *Bact. dysenteriae*, a highly pathogenic species, while least growth occurred in suspensions of *Staphylococcus albus*, a mildly pathogenic form.

Series III.

In this series seven chromogenic species of bacteria were investigated as a primary source of food for *Glaucoma ficaria*. The procedure of investigation was the same as in the preceding series.

Suspensions of the bacteria were prepared in sterile salt solution, as previously described. PRINGSHEIM'S medium alone was used as a control. The initial concentration of ciliates was 300 per cc., and the cultures were incubated for 48 hours. The results are expressed in table 3.

Table 3.

Growth of *G. ficaria* in suspensions of chromogenic bacteria; x_0, 300 per cc.

Species	Initial pH	Final pH	x/x_0	Transfers 1—15	Transfers 16—30
B. fluorescens	6.4	6.7	25.8	+	+
B. violaceum	6.6	6.8	9.3	+	+
B. rubidus	6.2	6.2	11.7	+	+
Bact. prodigiosus	6.3	6.6	8.3	+	+
M. phlei	6.4	6.4	3.0	—	
Sarcina lutea	6.4	6.4	4.9	—	
Sarcina aurantiaca	6.3	6.5	21.1	+	+
Control	6.2	6.2	0.7	—	

All the suspensions of bacteria in this series supported some growth of *Glaucoma ficaria*; however, the increase of the ciliates in the first transfer was not significant in suspensions of *Sarcina lutea* and *Mycobacterium phlei*, and subcultures on suspensions of these latter two species of bacteria were unsuccessful, the ciliates being dead at the end of the fourth transfer. This was true also for the control cultures. *M. phlei*, like *M. smegmatis* (Series I), forms large, compact aggregates of cells which cannot be ingested by the ciliates. No explanation is available at present for the failure of *G. ficaria* to grow in suspensions of *Sarcina lutea*. As indicated above, some growth occurred in the initial culture but the ciliates died off after a few transfers. This initial growth would seem to preclude any toxic effect produced by the bacterium. Furthermore, microscopic examination of the ciliates failed to show any abnormalities other than the usual diminution in size which is characteristic of starved individuals. Further attempts to maintain *G. ficaria* in suspensions of *Sarcina lutea* and *M. phlei* were all negative.

Dead bacteria as a source of food.

Series IV.

In this series attempts were made to grow *Glaucoma ficaria* in suspensions of dead bacteria. As in the preceding experiments suspensions of the bacteria were prepared in sterile PRINGSHEIM'S medium, but in this case the bacteria were killed by autoclaving.

Eleven species of bacteria were used. The initial count was 440 ciliates per cubic centimeter. After 48 hours incubation the ciliates were transferred to fresh media, subsequent transfers being made every two days for a period of 30 days and thereafter every seven days for a period of ten weeks. The results are summarized in table 4.

Table 4.

Growth of *G. ficaria* in suspensions of dead bacteria; x_0, 440 per cc.

Species	Initial pH	Final pH	x/x_0	Transfers 1—15	Transfers 16—25
B. subtilis	6.6	6.8	9.9	+	+
B. fluorescens	6.5	6.8	17.3	+	+
B. rubidus	6.7	6.7	12.8	+	+
Bact. lactis aerogenes	6.6	6.9	40.7	+	+
Bact. prodigiosus	6.8	6.9	13.8	+	+
Bact. coli	6.5	6.7	24.5	+	+
M. smegmatis	6.8	7.0	4.7	+	+
M. phlei	6.7	6.8	3.8	+	+
Staphylococcus albus	6.5	6.5	6.6	+	+
Spirillum rubrum	6.5	6.8	11.3	+	+
Sarcina lutea	6.7	6.8	9.4	+	+
Control	6.3	6.3	0.7	—	

The ciliates increased in all the experimental cultures of this series during the period of incubation, and successive transfers were successful in every case. Maximal growth of *G. ficaria* occurred in suspensions of dead *B. lactis aerogenes*, which also supported the greatest amount of growth in suspensions of living bacteria (table 1). An interesting feature of this series was that growth was maintained on suspensions of dead *M. smegmatis*, *M. phlei* and *Sarcina lutea*, the three species which gave negative results in suspensions of living bacteria (tables 1 and 3).

Series V.

HETHERINGTON (1933, 1934) reported that *Colpidium colpoda* would not grow in suspensions of carefully washed *Aerobacter aerogenes* and *Bacillus niger*; unwashed, however, both bacteria supported good growth of the ciliate. It was, therefore, thought advisable to investigate growth of *Glaucoma ficaria* in suspensions of washed bacteria. Suspensions of four species of bacteria were prepared in sterile PRINGSHEIM'S medium after being thoroughly washed four times in the manner previously described for washing ciliates. The initial number of ciliates was 490 per cc., and the

cultures were incubated for 48 hours. Sub-cultures were made at two day intervals as in the preceding experiments. Growth of the ciliates occurred in all cultures (table 5) and was maintained in the subsequent fifteen transfers, after which the experiment was discontinued.

Table 5.

Growth of *G. ficaria* in suspensions of washed bacteria; x_0, 490 per cc.

Species	Initial p_H	Final p_H	x/x_0	Transfers 1—15
B. subtilis	6.2	6.3	13.0	+
B. rubidus	6.4	6.8	6.8	+
Staphylococcus albus	6.5	6.7	11.0	+
Bact. lactis aerogenes	6.6	7.0	27.2	+
Control	6.2	6.2	0.8	—

Growth in relation to hydrogen-ion concentration.

Series VI.

Previously, the writer (MSS) found that growth of *Glaucoma ficaria* at different hydrogen-ion concentrations depended to a considerable extent upon the culture medium used. Similar results have also been reported for *Colpidium striatum* and *C. campylum* (ELLIOTT, 1933, 1935). In the present series growth of *G. ficaria* in relation to p_H of the medium was investigated, using suspensions of *Proteus vulgaris*, *B. fluorescens*, *B. mucosum capsulatum* and *B. prodigiosus* as food. In a preliminary experiment several samples of PRINGSHEIM's medium were adjusted to various hydrogen-ion concentrations and then autoclaved. After sterilization the p_H of the various samples had changed considerably, e. g., from p_H 4.4 to 5.1 and p_H 8.7 to 7.2. Obviously this medium was very slightly buffered and therefore unsuitable for use in this series. Other solutions were similarly tested and the following, in which the p_H changes during sterilization were minimal, was eventually used as a basic medium in this series.

KH_2PO_4 .	0.3 gm.
H_3BO_4 . . .	0.26 gm.
KH-phthalate .	0.46 gm.
Distilled water	1.0 liter.

Thick suspensions of each of the four bacteria were prepared in sterile 50 cc. samples of the above medium. The experimental

culture tubes for each experiment were then inoculated with 1 cc. of the corresponding bacterial suspension, the four experiments being run simultaneously. The p_H of the different sets of culture tubes ranged from p_H 4.0 to 8.6, and the initial concentration of ciliates was 660 per cc. After incubation for 48 hours, the final p_H was determined in each case, and the tubes were fixed for counting. The p_H of the culture tubes had changed but slightly (0.0—0.3) during the experimental growth period. The results are expressed as x/x_0 in figure 1.

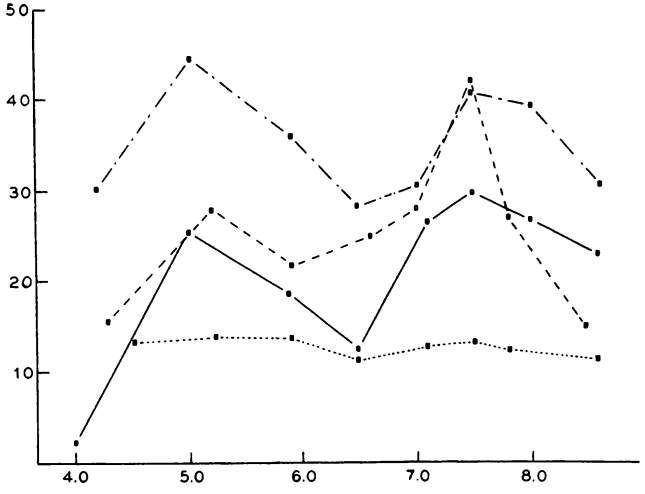

Fig. 1. *Glaucoma ficaria*, growth in relation to p_H; x/x_0 (ratio of final to initial concentration of organisms) plotted against initial p_H; in suspension of *Proteus vulgaris* (———), *Bacillus fluorescens* (- - - -), *B. mucosum capsulatum* (— · — · — · —) and *Bact. prodigiosus* (·····).

Growth occurred over the entire range investigated in each experiment and was bimaximal in suspensions of three of the bacteria with optima at p_H 5.0 and 7.4 in suspensions of *Proteus vulgaris* and *B. mucosum capsulatum*, and at p_H 5.2 and 7.5 in suspensions of *B. fluorescens*. Quite unexpected results were obtained when a suspension of *B. prodigiosus* was used as food. In this case the ciliates showed a remarkably constant growth over the p_H range investigated (4.5—8.6). A similar phenomenon has been reported by PHELPS (1934), who found that in suspensions of this same bacterium the division rate of *Paramecium aurelia* was comparatively unaffected by changes in the hydrogen-ion concentration between p_H 5.9 and 8.2.

Yeasts, mold spores and algae as sources of food.

Series VII.

A procedure similar to that in the preceding experiments was followed in determining the growth of *Glaucoma ficaria* in suspensions of yeasts. The experimental cultures were prepared by introducing *Saccharomyces cerevisiae*, *Sacch. ellipsoideus* and *Sacch.* sp. (containing a red pigment) into sterile PRINGSHEIM's medium. As in the preceding experiments the ciliates were washed four times before the cultures were inoculated. The initial count was 360 organisms per cc., and the culture tubes were incubated for 48 hours. Successive transfers were made as before. Moderate growth of the ciliates occurred in suspensions of *Sacch. cerevisiae* and sub-cultures have been maintained for over four months. Growth of the ciliates was negligible in suspensions of the other two species of yeast and in both cases the ciliates were dead at the end of the third transfer. Repeated attempts to culture the ciliates on suspensions of these two species of yeast have failed. However, successful cultures were later obtained using dead yeasts. Suspensions of *Saccharomyces ellipsoideus* and *Saccharomyces* sp. were prepared in PRINGSHEIM's medium and then killed by autoclaving. The initial number of ciliates was 440 per cc. After incubation for 48 hours growth of the ciliates on dead yeast was found to be better than in the suspensions of *Sacch. cerevisiae* (table 6) and these cultures have been transferred successfully for a period of three months. As in the preceding series transfers were made every two days for the first month and thereafter at intervals of 7 days.

Table 6.

Results using suspensions of yeasts; x_0, 360 per cc for the living yeasts and 440 per cc for the dead yeasts.

Species	Initial pH	Final pH	x/x_0	Transfers 1—15	Transfers 16—23
Saccharomyces cerevisiae	6.0	6.0	7.2	+	+
Sacch. ellipsoideus	6.2	6.2	1.5	—	
Sacch. sp. (red)	6.2	6.2	1.1	—	
Control	6.2	6.2	0.7	—	
Suspensions of dead yeasts					
Sacch. ellipsoideus	6.1	6.1	19.3	+	+
Sacch. sp. (red)	5.8	5.8	10.5	+	+
Control	6.2	6.2	0.8	—	

Series VIII.

A similar procedure was followed in establishing growth of *Glaucoma ficaria* with mold spores. Suspensions of the spores of *Aspergillus niger* and *Penicillium expansum* were prepared in sterile PRINGSHEIM'S medium. The fungi were cultured on agar plates and the spores were floated off by introducing sterile medium onto the surface of the cultures. The medium containing the spores was then removed to culture tubes by means of sterile pipettes. As in other experiments, the ciliates were carefully washed before inoculation. The initial and final p_H of both sets of culture tubes was 6.1, and the initial concentration of ciliates was 340 per cc. After 48 hours incubation the number of ciliates was 1600 per cc. in *Aspergillus* cultures and 1225 per cc. in the *Penicillium* cultures. This increase is small as compared with the growth of the ciliates in suspensions of bacteria; however, it was sufficient to permit successive transfers over a period of two months. Growth of the ciliates was also slight in the sub-cultures, and the consistently small size of the *G. ficaria* in these cultures indicates that the fungus spores are not a very favorable diet.

Series IX.

Pure cultures of *Chlorella* sp. also supported good growth of *Glaucoma ficaria*. Suspensions of the alga were prepared in PRINGSHEIM'S medium as in the preceding experiments. The initial concentration of washed ciliates was 245 per cc. The initial p_H of the culture tubes was 6.2 and the hydrogen-ion concentration did not change during the 48 hour incubation period. The ciliates increased to 2340 per cc. during the growth period, and these cultures have been maintained for a period of five months, during which 25 successive transfers have been made. There was evidence of an adjustment on the part of the ciliates to this diet of *Chlorella*, as indicated by the flourishing population of ciliates in the last few sub-cultures as compared with the moderate growth in the first few transfers. No evidence of a symbiotic condition was noted, the algae always being enclosed in the food vacuoles of the ciliate after being ingested.

Protozoa as a source of food.

Series X.

In this series suspensions of *Euglena gracilis*, *E. stellata*, *E. deses*, *E. klebsii*. *E. anabaena*, *Haematococcus pluvialis*, *Chlorogonium elongatum*, *C. euchlorum*, *Astasia ocellata*, *Astasia sp.* and *Chilomonas paramecium*

were used as food for *Glaucoma ficaria*. The flagellates were grown in peptone media and were washed in the same manner as the ciliates previous to introducing them into the culture tubes containing PRINGSHRIM's medium. The initial p_H of the different sets of experimental cultures varied from 6.1—6.5, and the initial number of ciliates was 247 per cc. The cultures were incubated for 48 hours, transfers were made to fresh media and the tubes were then fixed for counting. The p_H of the media had changed but slightly (0.0—0.1) during the growth period. In none of the cultures of this series was there a significant increase in the number of ciliates and in all cases sub-cultures failed. The experiment was repeated with the same negative results.

Since suspensions of dead yeast supported growth of *Glaucoma ficaria* even though living suspensions of the same yeast species would not, it was thought advisable to determine whether the same condition might exist in regard to the flagellates. Cultures of seven of the flagellates used above were killed by autoclaving, washed four times as before and then introduced into PRINGSHEIM's medium. These suspensions of dead cells were inoculated with washed ciliates and incubated for 48 hours. The initial number of ciliates was 450 per cc and the initial p_H of the different sets of culture tubes varied from 6.0—6.4. Growth occurred in all of the tubes (table 7), and has been maintained in subcultures for 9 weeks, transfers being made at two day intervals for the first 4 weeks and thereafter every seventh day.

Table 7.

Cultures of *G. ficaria* using dead flagellates as food; x_0, 450 per cc.

Species	Initial p_H	Final p_H	x/x_0	Transfers 1—15	Transfers 16—20
Euglena deses	6.4	6.6	8.6	+	+
E. stellata	6.2	6.4	8.2	+	+
E. gracilis	6.0	6.1	7.5	+	+
E. anabaena	6.4	6.4	9.1	+	+
Haematococcus pluvialis	6.4	6.5	6.7	+	+
Chlorogonium elongatum	6.4	6.4	4.0	+	+
Control	6.2	6.2	0.6	—	

Discussion.

The present investigation has shown that 21 species of bacteria, one yeast and an alga will support growth of *Glaucoma ficaria* in successive transfers for periods up to five months, the living food-organisms being suspended in a non-nutrive medium. Of the bacteria

used, *Bact. lactis aerogenes* was most satisfactory as food for the ciliates. Few of the previous investigators have used non-nutritive media in establishing mixed cultures of ciliates and other microorganisms. RAFFEL (1931) used a non-nutritive salt solution in maintaining *Paramecium aurelia* on a mixture of pure lines of *Strichococcus bacillaris* and *Bacillus candicans*, and JOHNSON (1933) likewise cultured *Oxytricha fallax* in suspensions of *Pseudomonas fluorescens* and *Bacillus subtilis* using a non-nutritive medium. Such a procedure is essential in the case of *Glaucoma ficaria* since this form may obtain nutriment solely through saprozoic means (JOHNSON, 1935/36). This is also true of several of the other ciliates used by earlier workers, e. g., *Colpoda steinii* (OEHLER, 1924), *Colpidium campylum* and *Glaucoma scintillans* (HETHERINGTON, 1933), *Paramecium caudatum* (GLASER and CORIA, 1933) and *P. bursaria* (LOEFER, 1934). With saprozoic ciliates, the use of a nutritive medium in preparing mixed cultures with bacteria does not give a true estimate of their ability to feed solely on the bacterial cells, since the ciliates may also receive nourishment either from the medium directly or from the products of bacterial action upon the medium.

Few investigators have succeeded in growing ciliates in media containing dead microorganisms. OEHLER (1919) obtained growth of *Colpoda steinii* on dead bacteria and dead yeast; E. and M. CHATTON (1923) cultured *Glaucoma scintillans* on dead bacteria; and GLASER and CORIA (1933) grew *Paramecium caudatum* in media containing either dead yeast or dead bacteria. In the present investigation *Glaucoma ficaria* has been cultured successfully on dead bacteria, dead yeasts and dead flagellates. In the case of *G. scintillans*, E. and M. CHATTON reported that growth of the ciliates would occur only after the culture media had previously been acted upon by the bacteria used. In other words if the bacteria were removed from their medium and transferred with the *Glaucoma* to a new medium, no growth of the ciliates took place. This would seem to indicate that the dissolved products of the activity of the bacteria have an important effect on the growth of this form. No such bacterial activity was essential in the present case or in the experience of OEHLER and of GLASER and CORIA, who killed the food-organisms and then added them to the culture medium.

Other workers have been less successful in attempts to culture ciliates on dead bacteria, e. g., LUCK, SHEETS and THOMAS (1931) used bacteria killed by a variety of chemical and physical means but could not obtain growth of *Euplotes taylori*; PHELPS (1934) likewise

failed in attempts to culture *Paramecium aurelia*, using similar methods, and HETHERINGTON (1934) found that heat-killed bacteria would not support growth of *Colpidium colpoda*. These latter three investigations involved isolation cultures, whereas the present investigation and those of OEHLER, CHATTON, and GLASER and CORIA were carried out in mass cultures. This latter method may to a large extent be responsible for the success of the investigators using it, and by its use many of the difficulties of other workers may be eliminated. This is true not only for growth of ciliates on dead microorganisms but also for growth in suspensions of living cells. Furthermore, the response of a ciliate in mass cultures gives a better index to the physiological characteristics of the species than can be obtained from observations on isolation cultures.

PHILPOT (1928) found that ordinarily cultures of *Bact. pyocyaneus* were toxic to *Paramecium aurelia*, *P. caudatum*, and *P. calkinsi*: however, the ciliates were able to develop an immunity to the toxicity of the bacteria. No such tolerance was developed by the ciliates to cultures of *Bact. enteritidis*, this bacterium always having a lethal effect upon the ciliates. The various species of pathogenic bacteria, including *Bact. pyocyaneus* and *Bact. enteritidis*, used in the present investigation had no apparent toxic effect upon *Glaucoma ficaria*, and in general were equally good as the non-pathogenic types for use as food. OEHLER (1924) reported that *Colpidium campylum*, *Colpoda steinii* and *C. cucullus* would live in pure cultures of *Bact. coli*, *Bact. fecalis alcaligenes*, *Staphylococcus aureus* and *Corynebacterium diptheriae*; and E. M. CHATTON (1923, 1925) cultured *Glaucoma scintillans* on *Bact. coli*. Apparently there is considerable difference between various species of ciliates in regard to their ability to utilize bacteria, the smaller *Glaucoma*, *Colpidium* and *Colpoda* being much less restricted in diet than the larger *Paramecium*. A similar difference has been noted in the growth of these ciliates in cultures on non-pathogenic types of bacteria.

When grown in suspensions of *Bacillus flourescens*, *B. mucosum capsulatum* and *Proteus vulgaris*, *Glaucoma ficaria* showed the bimaximal growth-p_H curve with maximal development at p_H 5.0—5.2 and p_H 7.6, while in suspensions of *B. prodigiosus* the division rate of the ciliates was approximately the same over the range investigated (p_H 4.5—8.6). PHELPS (1934) has reported a similar condition for *Paramecium anrelia* which also has a reasonably constant division rate from p_H 5.9—8.2 when grown in pure cultures of *B. prodigiosus*. This writer (1934, p. 161) makes the following statement concerning

this conditions: "by the use as in the present investigation of a pure line of bacteria the *Paramecium* are allowed maximum feeding of an adequate source of food at various H-ion concentrations, the differences in division rate are obliterated". However, the division rate of *Glaucoma ficaria* in suspensions of three other species of bacteria varied with changes in the hydrogen-ion concentration, a phenomenon which is normally characteristic of the growth of protozoa in general. Only in suspensions of *B. prodigiosus* did the division rate of this ciliate remain fairly constant at different hydrogen-ion concentrations. Therefore, it is obvious that the uniform division rates of *Glaucoma ficaria* and *Paramecium aurelia* in pure cultures of *B. prodigiosus* are due to the unusual effect of this bacterium rather than to the use of pure bacterial cultures or to an adequate food supply as suggested by PHELPS (1934).

The size of the food-organism unquestionably plays on important role in the growth of ciliates on other microorganisms. PRINGSHEIM (1928) found that algae and small flagellates such as *Polytoma* and *Chlorogonium* provided optimal development of *Peramecium bursaria*, while larger flagellates such as *Euglena* were less useful. The failure of *P. bursaria* to grow satisfactorily on *Euglena* is undoubtedly due to the fact that the latter are too large to be ingested easily by the ciliates. A similar condition probably explains the failure of *Glaucoma ficaria* to grow in pure cultures of *Saccharomyces ellipsoideus* and *Saccharomyces sp.* (red) and of the various flagellates used in the present investigation. However, when these yeast and flagellates were killed by autoclaving they provided a suitable culture medium for the ciliates. During the process of autoclaving considerable fragmentation and probably cytolysis of the cells takes place, so that the ciliates can then obtain food either by holozoic or saprozoic nutrition.

Summary.

Glaucoma ficaria was successfully cultured in suspensions of 21 species of living bacteria, one species of live yeast and one species of algae; in suspensions of 11 species of dead bacteria, 2 species of dead yeast and 6 species of dead flagellates. Since *G. ficaria* may obtain food saprozoically, suspensions of all food-organisms were prepared in a non-nutritive salt solution. Under such conditions, the pathogenic types of bacteria used apparently produced no toxic effects on the ciliates. The usefulness of yeast and small protozoa as food for *G. ficaria* depended largely upon the

size of such organisms, the larger forms not being ingested by the ciliates. In suspensions of *B. prodigiosus*, the division rate of *Glaucoma ficaria* was approximately the same from p_H 4.5 to p_H 8.6, while in suspensions of three other species of bacteria the ciliates showed the bimaximal growth-p_H curve with maximal development at p_H 5.0 bis 5.2 and at p_H 7.6.

Bibliography.

ANDREJEW, E. (1928): Über den Einfluß des Bodens als einem Regulierungsfaktor des Mediums auf die Kultur Paramecium aurelia. Arch. f. Protistenk. Bd. 63 p. 94—104.

AMSTER (1922): Ein neues Züchtungsverfahren für Protozoen. Zentralbl. f. Bakt. Bd. 89 p. 166—168.

BARKER, H. A. and C. V. TAYLOR (1931): A study on the conditions of encystment of Colpoda cucullus. Physiol. Zool. Vol. 4 p. 620—634.

BUTTERFIELD, C. T. (1929): A note on the relation between food concentration in liquid media and growth. Pub. Health Reports Vol. 44 p. 2865—2872.

CHATTON, E. and M. CHATTON (1923a): La sexualité provoque experimentalement chez un Infusoire, Glaucoma scintillans. Predominance des conditions des milieu dans san determinisme. C. R. Acad. Sci. T. 176 p. 1091—1093.

— (1923b): L'influence des facteurs bacteriens sur la nutrition, la multiplication et la sexualité des Infusoires. Ibid. T. 176 p. 1262—1265.

— (1925): L'action des facteurs externes sur la sexualité des Infusoires. Bacteries zygogenes et azygogenes Zygose Bacteriènne et zygose saline. C. R. Soc. Biol. T. 93 p. 675—678.

— (1927): Sur le pouvoir cytolytique immediat des cultures de quelques Bacteries chromogenes. Ibid. T. 97 p. 289—292.

— (1929): Les conditions de la conjugaison du Glaucoma scintillans en culture lethobacteriènnes. Action directe et specifique de certains agents zygogenes. C. R. Acad. Sci. T. 188 p. 1315—1317.

CUTLER, B. W. and D. V. BAL (1926): Influence of Protozoa on the process of nitrogen fixation by Azotobacter chroococcum. Annals Applied Biol. Vol. 13 p. 516—534.

GEISE, A. C. and C. V. TAYLOR (1935): Paramecium for experimental purposes in controlled mass cultures on a single strain of bacteria. Arch. f. Protistenk. Bd. 84 p. 225—231.

GLASER, R. W. and N. A. CORIA (1930): Methods for the pure culture of certain protozoa. Journ. Exp. Med. Vol. 51 p. 787—806.

— (1933): The culture of Paramecium caudatum free from living microorganisms. Journ. Parasit. Vol. 20 p. 32—37.

HARGITT, G. T. and W. W. FRAY (1917): The growth of Paramecium in pure cultures of bacteria. Journ. Exp. Zool. Vol. 22 p. 421—454.

HETHERINGTON, A. (1933): The culture of some holotrichous ciliates. Arch. f. Protistenk. Bd. 80 p. 255—280.

— (1934a): The role of bacteria in the growth of Colpidium colpoda. Physiol. Zool. Vol. 7 p. 618—641.

— (1934b): The pure culture of Paramecium. Science Vol. 79 p. 413—414.

JOHNSON, D. F. (1935/36): Isolation of Glaucoma ficaria KAHL in bacteria-free cultures, and growth in relation to p_H of the medium. Arch. f. Protistenk. Bd. 86 p. 263—277.

JOHNSON, W. H. (1933): Effects of population density on the rate of reproduction in Oxytricha. Physiol. Zool. Vol. 6 p. 22—54.

LOEFER, J. B. (1935): A simple method for the maintenance of pure-line mass cultures of Paramecium caudatum on a single species of yeast. Trans. Am. Micr. Soc.

LOSINA-LOSINSKY, L. K. (1931): Ernährungsphysiologie der Infusorien. Arch. f. Protistenk. Bd. 74 p. 18—121.

LUCK, J. M., G. SHEETS and J. O. THOMAS (1931): The role of bacteria in the nutrition of Protozoa. Quart. Rev. Biol. Vol. 6 p. 46—58.

LUDWIG, W. (1928): Der Betriebsstoffwechsel von Paramecium caudatum. Arch. f. Protistenk. Bd. 62 p. 12—40.

LUND, B. L. (1918): Toxic action of KCN on Paramecium and Didinium. Biol. Bull. Vol. 35 p. 211—231.

MICHELSON, E. (1928): Existensbedingungen und Cystenbildung bei Paramecium caudatum. Arch. f. Protistenk. Bd. 61 p. 167—184.

OEHLER, R. (1919): Flagellaten- und Ciliatenzucht auf reinem Boden. Arch. f. Protistenk. Bd. 40 p. 16—26.

— (1920): Gereinigte Ciliatenzucht. Ibid. Bd. 41 p. 34—49.

— (1921): Wirkung von Bakteriengiften auf Ziliaten. Zentralbl. f. Bakt. Bd. 86 p. 494—500.

— (1924 a): Weitere Mitteilungen über gereinigte Amöben- und Ciliatenzucht. Arch. f. Protistenk. Bd. 112—134.

— (1924 b): Geringte Zucht von freilebenden Amöben, Flagellaten und Ciliaten. Ibid. Bd. 49 p. 287—296.

PHELPS, A. (1934): Studies on the nutrition of Paramecium. Arch. f. Protistenk. Bd. 82 p. 134—163.

PHILLIPS, R. L. (1922): The growth of Paramecium in infusions of known bacterial content. Journ. Exp. Zool. Vol. 36 p. 135—183.

PHILPOT, C. H. (1928): Growth of Paramecium in pure cultures of pathogenic bacteria and in the presence of soluable products of such bacteria. Journ. Morph. Vol. 46 p. 85—129.

POWERS, J. H. and C. MITCHELL (1910): A new species of Paramecium experimentally determined. Biol. Bull. Vol. 19 p. 324—331.

PRINGSHEIM, E. G. (1928): Physiologische Untersuchungen an Paramecium bursaria. Arch. f. Protistenk. Bd. 64 p. 289—418.

RAFFEL, D. (1930): The effect of conjugation within a clone of Paramecium aurelia. Biol. Bull. Bd. 62 p. 244—257.

WEYER, G. (1930): Untersuchungen über die Morphologie und Physiologie des Formwechsels der Gastrostyla steinii. Arch. f. Protistenk. Bd. 71 p. 139—228.