(Columbia University Zoological Laboratory and Washington Square College Biological Laboratory.)

Studies in the life histories of Euglenida [1].

II. The Life Cycles of *Entosiphon sulcatum* and *Peranema trichophorum*.

By

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(With 8 figures in text).

Contents.

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1. Introduction.

Interest in the question of natural death in the Protozoa dates back almost to the time they were discovered. Ehrenberg in 1838 asserted that they were so simple in organization they did not

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undergo natural death, while DUJARDIN in 1841 maintained the existence of a definite life cycle terminating in death. O. F. MULLER in 1786 had already noted conjugation and interpreted it as a sexual act. WEISMANN (1883) stated that Protozoa do not grow old and do not die a natural death. But with the rocognition of the reorganization prozesses connected with conjugation, his theory was questioned by MAUPAS who in 1885—89 made efforts by means of isolation cultures to determine whether or not suppression of conjugation would result in ageing and death. Since his experiments the problem has been attacked by many workers, dealing mainly with Infusoria.

Their findings have resulted in two different views in regard to the life cycles of Infusoria. CALKINS believes that there is a definite life cycle for ciliates and that natural death is inherent in them under natural conditions. WOODRUFF and others believe that death is due to poor cultural methods (a faulty environment) and that as cultural conditions are improved the life cycle will disappear. CALKINS thinks that the reorganization processes of conjugation and endomixis cause a renewal of vitality; WOODRUFF (1926) asks: „Can unicellular animals reproduce indefinitely without recourse to fertilization?“ He asserts they can, if subjected to a favorable environment.

There is little published concerning the life cycles of protozoa other than ciliates. BĚLAŘ (1924) has followed the life history of one of the Sarcodina, *Actinophrys sol*, a heliozoon, for thirty two months. He obtained 1244 generations in this time, and since there were no fertilizations in the direct line, and no waning vitality, he concludes that the organism can live indefinitely under proper environmental conditions, reproducing by binary fission. In the Mastigophora there is likewise one published account of a life cycle; that of *Eudorina elegans*, worked out by HARTMANN (1921). He followed the history of this species by the isolation culture method for about five years, and his longest series lived for 1300 generations. He perfected his technique until he obtained a constant, favorable environment, so that there was practically no fluctuation in the latter part of the life history of his organism. Earlier fluctuations were attributed to unfavorable environmental fluctuations. From his results, HARTMANN concluded that *Eudorina* could live indefinitely without conjugation or an equivalent fertilization process.

It is generally understood that the great majority of flagellates are capable of maintaining their life cycle indefinitely without

conjugation. In the class Phytomastigoda only one of the six orders, the Phytomonadida, has well known sexual reproduction. *Eudorina* belongs to this group, reproducing sexually by anisogamy, sperm and eggs being developed in male and female colonies. In the Euglenida there is a questionable case of isogamy in one genus, *Copromonas* (DOBELL, 1908; BERLINER, 1909), while HAASE (1910) has described intranuclear gamete formation with conjugation in *Euglena sanguinea*, which has not been confirmed. In the Dinoflagellida sexual processes are reported in a few cases which lack confirmation. In the other three orders sexual reproduction is unknown. No reported instance of fertilization in the class Zoomastigoda has been verified.

The reorganization processes attendant on cell division are well known in the Mastigophora generally. Division may occur in the active state in some forms and in the encysted state in others. But while the mechanism of division is well known in many forms where it occurs in the encysted state, there has been no encystment in the life cycle of Eudorina, and in no flagellate has the vitality of the organism been tested after exystment.

The isolation cultures described in this paper were carried for the purpose of testing the supposed continued vitality of non-green flagellates in which neither conjugation nor encystment has been described. Two forms were used; *Entosiphon sulcatum* which is a saprophytic form, and *Peranema trichophorum* which is known to live holozoically, at least to some extent. After determining under what environmental conditions the animals could be carried as laboratory cultures, an answer was sought for the following questions:

1. Is a given level of vitality maintained, or are there changes in the vitality of the organism? If there are changes, what is their nature and cause?

2. Does conjugation and fertilization occur?

3. If there is neither fertilization or conjugation, is encystment with attendant reorganization a part of the life cycle?

4. Is the observed reorganization occurring at each division sufficient to maintain the vitality of the organism?

2. Methods.

At the beginning of the work *Entosiphon* only was used, and the animals were carried on the synthetic medium of PETERS (1921), on which they had thrived as mass cultures, for some time. This medium was made up in liter quantities, and from this small

amounts were transferred to test tubes two or three times a month. These were plugged with cotton, capped with lead foil and sterilized in an Arnold sterilizer, small amounts being used daily. This medium was used until October 30, 1925, when it was discarded for one of cracked wheat in spring water (LACKEY, 1927), which had been tested and had proved successful.

The cultures were maintained in embryological watch glasses, the salt cellar type, with polished and ground concavities. These were kept in moist chambers. Cultures kept in the room were subject to diffuse daylight, but never to strong light, except during examination. All glassware was carefully washed and was soaked overnight in distilled water before draining for use. Isolations were made under a binocular, using 10X oculars and 40 mm. objectives. Because of the very small size of the organisms, it was found advisable to use as small a drop of water as possible in which to grow them. Evaporation had to be watched very carefully. If questions arose concerning transfers, the one in question was discarded and a new one made.

Temperature was not controlled until January, 1926, when certain lines were transferred to an incubator. No control of pH was made, and it fluctuated between 6,8 and 7,6. Throughout the work an effort was made to keep all technical methods uniform.

3. History of Cultures.

a) Entosiphon Series.

On April 5, 1925, three series, EW, EV and EX, of *Entosiphon sulcatum* were started from the progeny of a single individual picked out of the mass culture previously referred to. On November 10, 1925, three other series, HE, 1-HE, and 2-HE, of *Entosiphon sulcatum* were similarly started. These came from an individual of a mixed culture of protozoa collected by Dr. ALFRED HUETTNER of Columbia University in Van Cortland Park, New York City. Many other series were started from time to time during the course of the work, but they all came from one or another of these two strains. Table (p. 132) one gives the history of the majority of the series experimented upon.

From the above table it is seen that the longest life of any isolation culture of *Entosiphon* was 634 days. Series EW and EV-EVC were discontinued at the end of this lengthy period, EW in the 947th generation, and apparently in full vigor. Figure 1 is the graph showing the daily division rates of the four lines of

Table One.

Length of life, fission rate per ten day period, and fate of all series of Entosiphon.

Series	Days of Life	From . . . . . To	Generations	Avg. fissions per 10 days	Fate
EW	634	Apr. 15, '25 — Dec. 28, '26	947	14,96	Discontinued.
EV	460	„ „ „ — July 7, '26	603	13,10	Killed.
EX	200	„ „ „ — Oct. 21, '25	290	14,50	Died.
EVA	198	Apr. 16, '25 — Oct. 20, '26	270	13,50	Died.
EVAA	95	Feb. 2, '26 — May 7, '26	133	13,30	Died.
EVB	157	„ „ „ — July 8, '26	289	18,40	Died.
EVC	330	„ „ „ — Dec. 28, '26	524	15,87	Discontinued.
EVD	190	„ „ „ — Aug. 10, '26	224	11,78	Killed.
EV-EVC	634	Apr. 15, '25 — Dec. 28, '26	905	14,27	Discontinued.
2-HE	412	Nov. 10, '25 — Dec. 26, '26	514	12,47	Discontinued.
2-HEA	107	July 8, '26 — Oct. 22, '26	81	7,57	Discontinued.
2-HEB	178	„ „ „ — Dec. 26, '26	243	14,12	Discontinued.

this series averaged for ten day periods covering the twenty one months of its life. Its average rate for the first ten day period was 17,25. Its highest division rate for any ten day period was 20,00, a figure attained only once. The lowest division rate for any ten day period was 5,00, between December 25, 1925, and January 3, 1926, at which time the room was so cold that most of the isolation cultures were lost. During this time line 1 of this series did not divide once.

Fig. 1 shows a rather large fluctuation of the division rate for this series, but this is largely attributed to temperature variations. In the latter part of October, 1925, the fluctuation is due to the food medium being unsatisfactory in some way. The supply had been made several months previously, and probably deteriorated with age. At any rate, when the cultures were transferred to a cracked wheat medium, the division rate at once went up.

December 22 to 31, 1925, was a period of cold and inattention for the cultures. In this ten day period the average division rate was only 6,50; the room was cold until about January 4th and the division rate remained low, an average of 8 for the first ten days of January. On the 10th, the series was transferred to an incubator and the division rate went up to 15,25. There was very little change in the division rate for the next 170 days of January 10, 1926 to June 27, 1926, but on July 1st, the cultures were all put in a heavily iced refrigerator for a period of six days. For the

six days there was an average of only four divisions and for the first ten days of July, including four days when the temperature

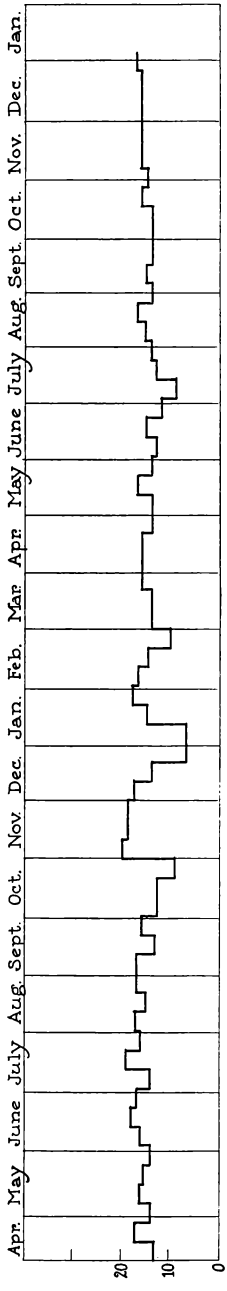

Fig. 1. Graph showing the fission rate per ten day period of *Entosiphon sulcatum*, Series EW during the twenty one months it was maintained.

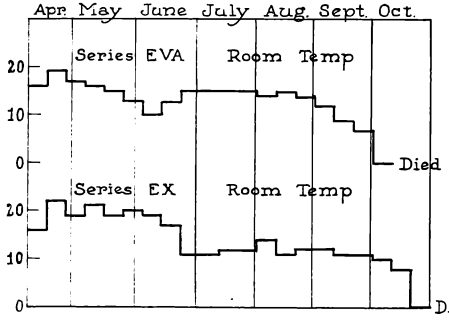

Fig. 2. Graphs showing the fission rates of *Entosiphon sulcatum*, Series EX and EVA.

rose somewhat, the average division rate was 7,50!

Thereafter the series was kept at room temperature until December 26, 1926, and again maintained a very equable division rate. Its division rate from October 15th, 1926 to December 13th, 1926 remained constant except for one period at an average of 16,00 divisions per ten day period. From October until the end of the experiment this series was kept in a tight cabinet inside the writer's laboratory, and there was relatively little fluctuation in temperature Variations in the division rate for the last nine months are small and point to a practically constant, favorable environment.

On April 6, 1925 a daughter series, EWA was taken from EW, The history of this series is shown in Fig. 2. This series lived for 198 days, the last survivor dying on October 20, 1925, in the 270th generation. From a high division average for the second ten day period it shows a gradual decline until death ensues. This graph is like those plotted for the life cycles of

many ciliates, except for a too quick decline. Death in this case was clearly due to faulty environment, as proved by the case of its parent series. The unfavorable factor in the environment was food as proved by the case of its parent series. Series EX, also shown in Fig. 2, shows a similar history, dying six days later in the 290th generation. Fig. 3 shows the history of series EV and three daughter series. The parent series was always kept at room temperature except July 1st to 8th, 1926, inclusive, and maintained a high average division rate until it was lost by careless handling in moving the refrigerator July 8th. Three times during its early history it uveraged 20 divisions per ten day period, but its division rate during the time it was carried on the synthetic medium was generally lower than while it was carried on wheat. There is no evidence of waning vitality in the graph of its life cycle. Its lowest division rate, 6,00 per ten day period, is coincident with the low division rate of all series during the cold of the Christmas holidays of 1925.

On February 2, 1926, five daughter series EVAA, EVB, EVC, EVD and EVN were started. The first, EVAA died at the end of 95 days, in full vigor, and with no depression of its division rate, and no reason can be assigned for the death of the series. Series EVN, maintained as a single line, was kept in an incubator at 26° C. It lived only 47 days. Its division rates for the 4,7 periods were 20, 18, 21, 16 and 10 respectively, then it suddenly died. The history of EVD, EVB and EVC are shown in Fig. 3.

Series EVD, carried for 40 days at room temperature, averaged 14,75 divisions for this time. Then it was put in the refrigerator for 120 days, at a temperature fluctuating between 11° and 14° C (a drop from about 21° C). Its division rate at once dropped from 18,00 to 9,00 averaging 8,41 for the 12 ten-day periods. At the end of this time it was put in the incubator at 26° C and the division rate rose from 7,25 to 16,00. For the next 40 days it averaged 17,25 divisions per ten day period, and was then accidentally lost. The sister series EVB, had a much higher division rate. Kept in the incubator from the beginning, its division rate for the first ten day period, was 20,25, and later it went to 22,00 and 23,00. At the end of 140 days it declined from 20,00 to 16,25, then to 8,00, and died abruptly at the end of 157 days. Its average division rate for the entire sime was 18,40. The third series EVC, carried altogether in the incubator during its life, except for the last six days of July, had an average division rate of 15,75 for 33 ten-day

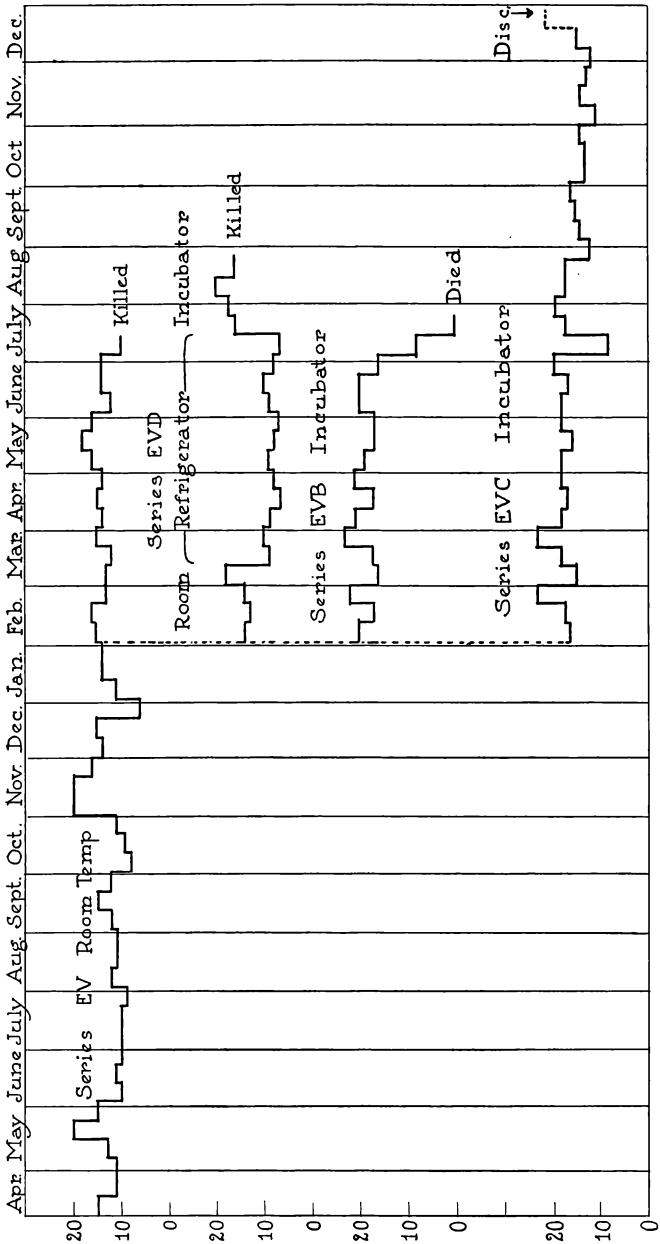

Fig. 3. Graph showing the fission rate of *Entosiphon sulcatum*, Series EV, and its daughter Series EVD, EVB and EVC.

periods. After August 20, 1926 its division rate was somewhat lower than previously. For the 190 days up to that time (excluding

the ten days of July 3rd to 11th.), its average rate of division was 17,94; for the 126 days after this until the time it was discontinued its average division rate was 13,88 per ten-day period. In the final six days of its life it averaged 13,00 divisions. From Fig. 3 one would infer that it was in a gradual decline, but this final figure would indicate a value of 21,00 for the entire ten days. As far as visual examination indicated, the culture was in a thriving conditions.

Fig. 4 gives the history of one parent and two daughter series of Entosiphon of a different strain than those heretofore spoken of, the Van Cortland Park strain. Three series of this strain were

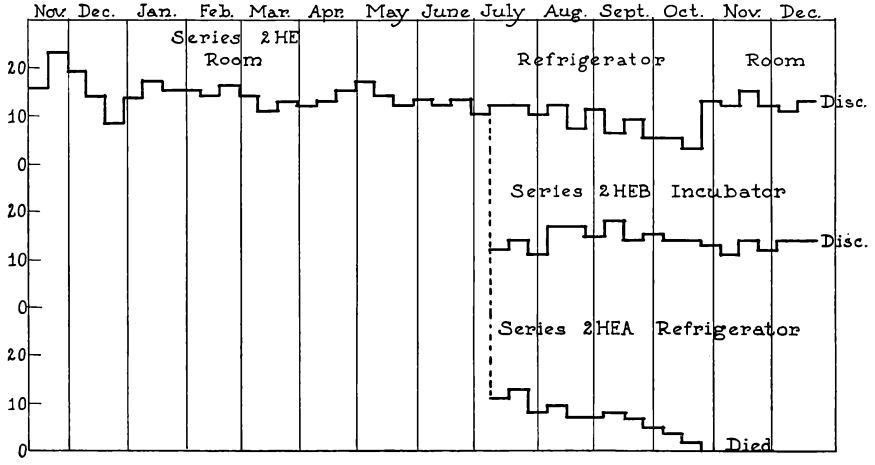

Fig. 4. The fission rates of three series of *Entosiphon sulcatum* under diverse conditions. Series 2-HE and daughter Series 2-HEB and 2-HEA.

originally started on November 10, 1925, of which one soon died from no discernible cause, and another died in the cold weather of December—January.

Series 2-HE was kept at room temperature until July 1, 1926 and during that time showed wide fluctuations of its division rate. A slight downward trend, from a high rate of 22,75, was evident but after it was placed in the refrigerator the downward trend became very pronounced. Between October 16th and 25th, 1926, one of the lines died, one did not divide, one divided once and the fourth twice. Fearing the series would die out altogether it was moved into the room and the division rate at once went up to 12,75, and remained high until the end. Series 2-HEA, similarly kept in the refrigerator from July 8th, had an initial value of

11,00, then went to 13,00 but after that steadily declined, the last individual dying October 22nd. A sister series, 2-HEB, kept in the incubator did not die or decline but maintained a high division rate, 14,00, almost twice that of the series which died.

This is a brief history of the principal series of the *Entosiphon* cultures. Summarizing the histories of these series, we find:

1. Two series, EW and EV-EVC thriving and maintaining their division rate after 633 days of isolation culture.

2. One series, 2-HE, of a different protoplasmic strain, thriving and maintaining a division rate approximately equal to that of series EW and EV after 411 days of continuous isolation culture.

3. No indication of a declining division rate for these three series.

4. Two series, EX and EVA, died after rather sudden drops in the division rate, death being due probably to faulty cultural methods (poor food). These series died at the end of 200 and 198 days.

5. One series, 2-HEA, died at the end of a sharp decline in division rate, after 107 days exposure to low temperature.

6. One series, 2-HEB, thriving and maintaining a high division rate after 172 days of continuous isolation culture in an indubator.

7. One series, EVB, maintaining a high division rate for 140 days in the incubator then suddenly dying after a sharp decline in division rate.

b) *Peranema* Series.

On October 27th, 1925 four series AP, BP, CP and DP, of *Peranema* were started. These were all daughter individuals from a single specimen isolated three days before which originally came from the same source as the cultures of *Entosiphon*. After isolation of these four sister series no change in cultural methods from those used for *Entosiphon* was made; they were given the same food medium and handled in the same manner as the *Entosiphon* cultures were. After three weeks however, series DP was discontinued, due to pressure of work.

Each of the original four series started with a high division rate in October. From figure 5, series BP is shown to have had an average division rate for its first ten day period of 7,00 and AP (Fig. 6) of 10,00 while the value for DP, the discontinued culture, was 9,00. All of these initial high division rates speedily dropped to lower levels. This initial high value might have been a hold-

over of a division rate common to the mass cultures due to some such factor as the occasional use of *Entosiphon* as food, not occurring in the isolation culture.

Only one of the original series of *Peranema* was in existence at the close of this phase of the work, in December, 1926; series CP, of which three daughter series were alive and thriving. Table 2 shows the histories of all series.

Table Two.

Showing series of *Peranema*, length of life, generations and average number of divisions per ten day period.

Series	Days of Life	From . . . . . To	Generations	Avg. fissions per 10 days	Fate
CP	369	Oct. 27, '25 — Oct. 30, '26	129	3,48	Died Out.
CPA [1])	170	July 3, '25 — Dec. 19, '26	61	3,58	Discontinued.
CPB [1])	150	July 23, '25 — Dec. 19, '26	59	3,93	Discontinued.
CP to July 3 + CPA [1])	420	Oct. 27, '25 — Dec. 19, '26	160	3,80	Discontinued.
CP to July 23 + CPB [1])	420	Oct. 27, '25 — Dec. 19, '26	167	3,97	Discontinued.
CP to July + CPB to Nov. + CPBA	420	Oct. 27, '25 — Dec. 19, '26	171	4,07	Discontinued.
BP	190	Oct. 27, '25 — May 3, '26	78	4,10	Died out.
BPA [2])	240	Nov. 13, '25 — July 8, '26	123	5,12	Killed.
BPAA	152	Dec. 8, '25 — May 8, '26	116	7,73	Died Out.
AP	130	Oct. 27, '25 — Feb. 4, '26	64	4,92	Died Out.
APA [2])	240	Nov. 13, '25 — July 8, '26	98	4,08	Killed.
APB	139	Dec. 8, '25 — Apr. 25, '26	96	6,85	Died out.
APBA	210	Jan. 7, '26 — Apr. 26, '26	83	7,54	Killed.
APB to Jan. 7 + APBA	140	Dec. 8, '25 — Apr. 26, '26	105	7,50	Killed.

[1]) Actually discontinued as thriving cultures 4 days later.

[2]) Actually lived 238 days, when killed in moving refrigerator. Correction made.

The series which lived the longest was CP — CPB-CPBA (Fig. 7) which lived 430 days and was discontinued in the 172nd generation. This series had an initial ten day division average of 12,00, and its average for the 43 periods was 4,00. The parent series, CP, lived 370 days, dying in the 129th generation. Its average division value was 3,48 per ten day period. This series was kept at room temperature for its first 250 days and lived through 99 generations in this time. After July 3rd it was kept in the refrigerator for 110 days, until it died out. There was an average of 30 divisions

per line in this time, or 2,72 per ten day period. Fig. 6 shows a gradual decline during this time, but the 110 days preceding July 3rd from March 15th had a lower average division rate, 2,54 per ten day period. For the first 110 days of its life, until

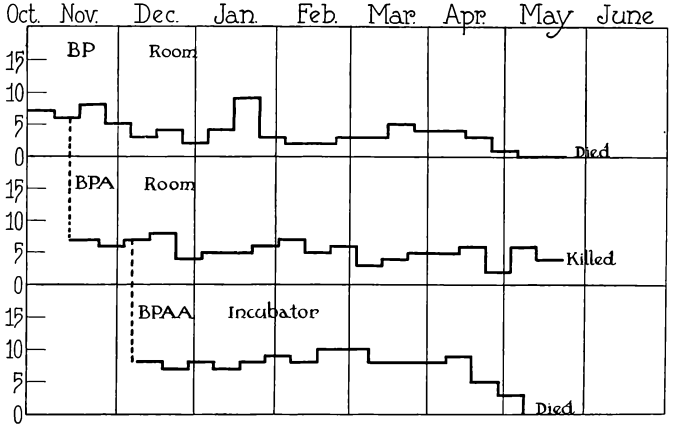

Fig. 5. Graph showing fission rate per ten day period of *Peranema trichophorum*, Series BP and its daughter Series BPA and BPAA.

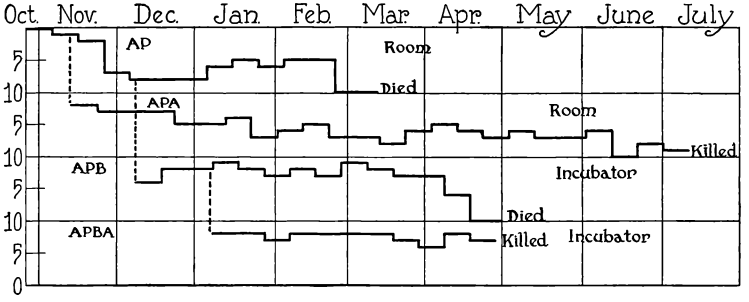

Fig. 6. Division rates of Series AP, and daughter Series APA, APB, and APBA, *Peranema trichophorum*.

February 12th this series had an average division rate of 6,00 per ten day period.

Its daughter series CPA, was kept in the refrigerator from July 3rd. to October 21st. It shows a gradual decline in its division rate also, but averaged 3,18 divisions for the 110 days as compared with 2,72 for the parent series. In the final ten days of this time line a divided twice, lines b and c once each and the individual in line d did not divide. The culture was then brought out into the room and at once the division rate went up to 7,00 and it

averaged 4,33 until discontinued in December. The second daughter series CPB, taken from CP on July 23rd, was kept in the room continuously and averaged 3,93 divisions per ten day period. From figure 7 there seems to be evidence of a gradual decline in the division rate, but it shows a recovery, and the division rate of CPBA, its daughter series is also high.

Fig. 5 shows the history of series BP and its two daughter series BPA and BPAA. The first two were kept at room temperature, while BPAA was kept in a incubator at 25° C. The highest average number of divisions per ten day period ever reached by either of the first two was 9,00 for BP. This series passed through a period of gradual decline, ending in death. Its daughter series never showed a decline except for brief periods. Its final value of 3,00 was forced by putting it in the heavily iced refrigerator during the enforced absence of the writer for 5 days in July 1926. Its death was due to an accident (moving refrigerator) mentioned above and was very unfortunate, for its average division rate, 5,12 per ten day period had been more consistently maintained than either AP or CP, the other two series which had lived to this date.

BPAA (Fig. 5) showed a much higher division rate than the parent series, BP, but this was terminated by a sudden decline and death. There is almost an exact parallel between its history and that of APB (Fig. 6) which was also an incubator culture. In both these series the high division rate was consistently maintained until the last twenty to twenty five days. The individual animals ef the incubator series did not attain quite the size of those kept at room or refrigerator temperatures. The size of these latter was often conspicuously large; their cytoplasm was filled with black refringent spherules as if large amounts of reserve food materials were stored up. The cytoplasm of the incubator cultures was usually rather clear, like that of mass cultures which had been kept for a long time. A temperature of 25° is evidently too high for this organism. A daughter series of APB (APBA) had not died at the same date and gave no evidence of a decline, but it was lost through accident.

Series AP (Fig. 6) started with a high division rate, underwent a rapid decline and died at the end of 140 days. One individual lived 23 days before it died. A daughter series, APB, also started with a high division rate and passed through a gradual decline. It had not died at the end of 240 days but was killed at the end of that time by accident (moving refrigerator). It did not show a

noticeable depression period during February-March or May as series CP did.

Summarizing briefly the histories of the *Peranema* series, we find:

1. One series, CP, after a noticeable depression period earlier in its history was alive and thriving (through three daughter series) after 14 months.

2. Two series, BP and AP, themselves underwent gradual depression of the division rate ending in death, but:

3. A daughter series of BP, BPA, did not show a great decline and was a thriving culture when it was killed at the end of 8 months; and a daughter series of AP (APA) while showing a gradual decline was still alive after 8 months.

4. Two series, BPAA and APB, kept in an incubator at 25° C maintained a high average division rate for about 5 months, but showed a sudden decline and death at the end of that time; a third series APBA under similar conditions had not shown any evidence of a decline when it was killed after 5 months.

5. Series CP and CPA were transferred from a room temperature of about 20° C to a refrigerator which ran between 11° and 15° C. Series CP at once showed a declining division rate and died after 4 months. Series CPA also showed a decline and was taken out of the refrigerator when at the end of 4 months its division rate had gone down to 1,00 in ten days.

4. Discussion.

a) Intrinsic Variations of Division Rate.

No categorical answer may be given to all four of the questions asked in the beginning of this work. But a discussion of the results obtained with the cultures may be enlightening enough to to serve as answers to all of them.

Unless there are intrinsic causes of variations in the vitality of a protozoön such as rhythms or depression due to the need of conjugation or endomixis, the vitality of that protozoön should express itself in a fission rate whose variations would be determined solely by environmental conditions. Once this environment becomes adverse the fission rate should fall, and if conditions become bad enough, the organism should cease to divide, and die.

To fully test such an assumption it is necessary that cultural methods be perfected to a very fine point, and constancy is practically impossible of attainment. The environment in which the cul-

tures of *Entosiphon* and *Peranema* were carried is certainly not a natural one, and it is not known in what respects it differs from a natural one but it is an environment which lends itself to being maintained with a fair degree of constancy. In it both *Entosiphon* and *Peranema* tend to maintain given levels of vitality. While some of the series died out during the course of the work, others were accidentally lost, and some were purposely stopped, three of the original series of *Entosiphon*, and one of the original series of *Peranema* were alive at the end of the work and were dividing at about the same rate as the beginning. It should be noted that *Peranema* when taken from a mass culture has a high initial division rate which drops in the isolation cultures, during about thirty days, to a relatively low division rate which is thereafter maintained, and shows no further evidence of a decline.

No one level of vitality has been long maintained in any series, but we have demonstrated that this is due to environmental conditions. *Entosiphon* series EW showed no deviation in its division rate for the five ten day periods prior to December 15, 1926, and during the 170 days prior to that date the lowest division rate for a ten day period was 13 and the highest 17, which shows a very slight fluctuation. RICHARDS and DAWSON (1927) have criticized the histogram plot commonly used in plotting protozoan life cycles, as misleading, and it may often be. But if we look at figure 8, which gives the daily division rate of series EW as a running average, in comparison with the ten day averages for the fifty days from October 26th to December 14th, 1926, we see that the daily variation is so small as to be almost negligible in this case.

Maintenance of a steady division rate was expected in both *Peranema* and *Entosiphon* because no conjugation or encystment has ever been reported for either of these organisms. In all groups of protozoa which have been investigated, there have been found some in which the reorganization processes of cell division have seemed adequate for maintenance of the protoplasm in a state of unimpared vitality. Such an adequacy is the explanation offered by CALKINS (1926) for the long cultures of *Eudorina elegans* and *Actinophrys sol* carried by HARTMANN and BĚLAŘ, and for the long maintenance of pedigreed cultures of *Paramecium calkinsi* vithout conjugation or endomixis by SPENCER. DAWSON's three year culture of *Bhepharisma* probably belongs in this group. These organisms have shown no signs of a waning vitality at the time the work on them was

reported, and it is highly probable that the cultures of *Entosiphon* and *Peranema* present a parallel case.

With but few exceptions the life cycles of pedigreed cultures of Infusoria show minor elevations and depressions of the fission rate, due to some unknown factor in cell metabolism, from which recovery is autonomous (Woodruff, 1905). Thus Calkins (1902) found such rhythms in the life cycle of *Paramecium caudatum* and Woodruff in *Oxytrichia fallax* and *Pleurotrichia lanceolata* (1905) and *Paramecium aurelia* (1904). Spencer (1924) by her work on *Paramecium calkensi* has proved that rhythms may occur independently of both temperature and endomixis, and thinks the cause of them to be intrinsic. Richards and Dawson (1927) do not believe that rhythms are intrinsic, and by their methods of the analysis of the division rates of ciliates, they show that there is a single seasonal rhythm, with a minimum in July, for *Paramecium aurelia*, *Blepharisma undulans*, and *Histrio complanatus*. They believe that constant culture methods will remove rhythms from the protozoan life cycle. Dawson (1926) obtained fluctuations in the life cycle of *Histrio complanatus* which he did not interpret as due to rhythms, but to environmental changes.

There is no evidence for rhythms in the cases of other protozoa than ciliates. Bělař found sharp fluctuations in the life history of his isolation cultures of *Actinophys*, but these are evidently due to changes in environment and not to rhythms. Hartmann had great difficulty in establishing uniform conditions for *Eudorina*, and his first isolation culture died at the end of a depression period due to an unfavorable environment. Under his best conditions — the last hundred days — a colony would reproduce itself every 4, 5, or 6 days. This meant five cell divisions in such a period, as every cell of the 32 cell colony divides 5 times to produce a new colony. He reckoned colony formation as the life of a single generation, and the shortest life of any generation in his cultures was three days, between July 10th and 13th 1915. In February-March, 1917, there was a period of twenty-six days between generations. His cultures showed a depression period about the end of the year for four out of five years, the occurrence of these periods being about as follows:

Oct. 29, '15 to Jan. 2, '12 No generations. First series died.
Oct. 7, '16 to Apr. 12, '17 Seven generations in this time.
Oct. 14, '17 to Feb. 22, '18 Four generations in this time.
Oct. 11, '18 to Jan. 6, '19 Four generations in this time.

But by November 14th, 1919, he had so perfected his methods of control that from that time no longer than seven days was ever

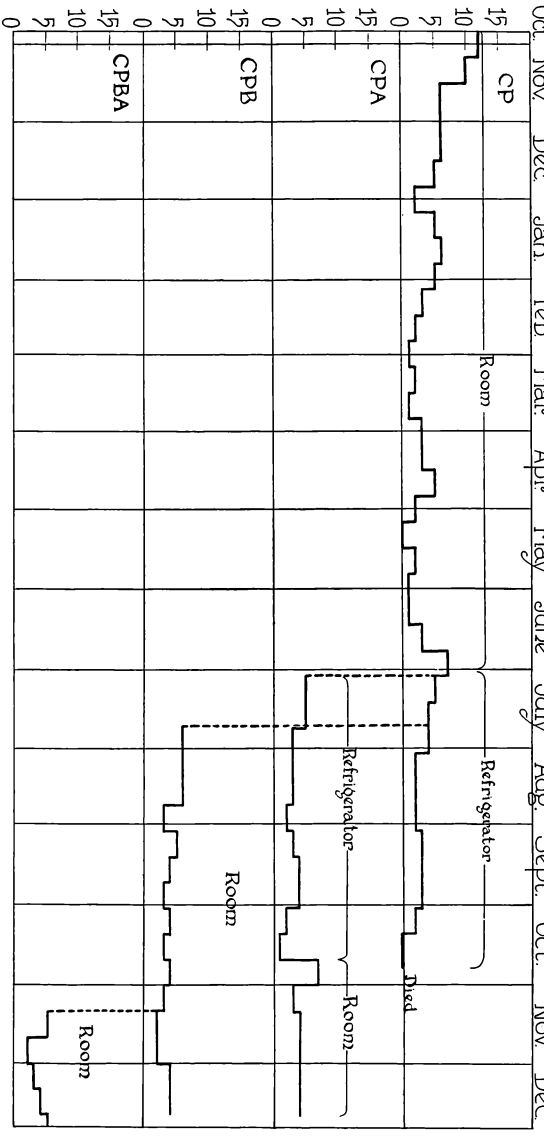

Fig. 7. Fission rates of four series of *Peranema trichophorum* during a period of fourteen months. Series CB, and daughter Series CPA, CPB, and CPBA.

needed to obtain a new generation. The depression periods encountered up to that time were clearly not rhythms, and removal of

the cause in the environmental conditions removed the depression in the division rate.

In the cultures of *Peranema* and *Entosiphon* there are fluctuations in the division rate, but they are mostly small, and are not considered of enough importance to be called rhythms. Thus in *Peranema*, series CP (Fig. 7) shows elevations of the division rate in January, April and July. There is no repetition of them at the same time in the other series and they are small fluctuations. Control of the food of this organism is difficult as it is a partially holozoic organism. With a saprophytic organism such as *Entosiphon*

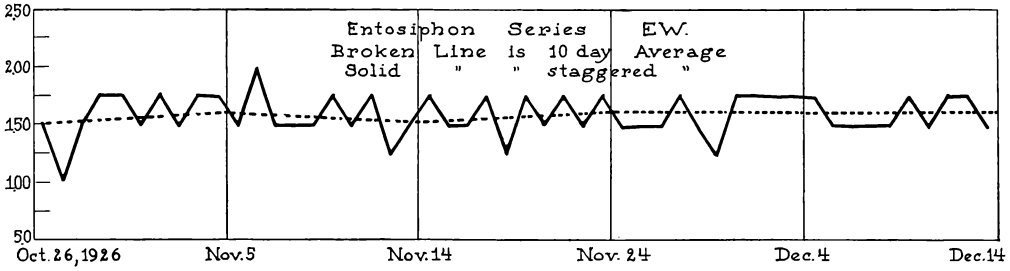

Fig. 8. Comparison of staggered and ten-day averages of the fission rate of *Entosiphon sulcatum*, Series EW, from October 26, 1926 to December 14, 1926. Four lines of the Series are averaged.

a closer control of all conditions should be possible and any fluctuations of the division rate ought to be accountable. Fig. 1 shows three pronounced depressions in the life history of series EW, and all three can be traced to a cause. Poor food was the cause of the first one. At this time the organisms were being carried on the synthetic food medium of PETERS, previously referred to, and every series was in a depression. Fig. 2 shows that series EX and EVA died when kept in this food medium, and all others which were transferred to the cracked wheat medium showed a complete recovery. Similarly, all of the series of both organisms showed sharp drops in the division rate at the end of December and the first of July, when they were subjected to low temperatures.

Practically all of the other series of *Entosiphon* show little or no fluctuations of the division rate other than those accounted for below, as due to changes in temperature. Furthermore it is shown that when conditions are kept as constant as possible, there are very few changes in the division rate. It is therefore concluded that rhythms do not exist in the life history of these organisms.

b) Temperature Effects.

Temperature can be shown to have a very marked effect on the division rate. Few workers have dealt with this factor in the division rate of protozoa. MAUPAS (1888—1889) kept careful records of the temperatures at which his cultures lived and gave a table showing the division rate of each one of fifteen ciliates at various temperatures in 24 hours.

5—10° C	*Stylonichia*	*pustulata*	Divided	1	time
10—15 „	„	„	„	2	„
15—20 „	„	„	„	3	„
20—24 „	„	„	„	4	„
24—28 „	„	„	„	5	„

The division rates as listed by MAUPAS show a wide fluctuation from *Stentor* to *Glaucoma* or *Leucophrys*, and while it is not known at what point in the life cycles his data was made for each species, a fact which might make considerable difference in certain cases, it is evident that the fission rates of various species are widely different at a given temperature. He gives a table of the division rate of the progeny of a *Stylonichia pustulata* ex-conjugant which shows a steady increase in the division rate, as the temperature increased and maintained that the division rate of ciliates remains constant after conjugation.

CALKINS (1902) noted the temperatures at which he kept isolation cultures of *Paramecium caudatum*, and found that he got fluctuations in the division rate independently of temperature variations and these he concluded to be inherent. WOODRUFF and BAITSELL (1911) made a careful study of the effect of temperature variations on the division rate of *Paramecium aurelia*. They pointed out that BOROWSKY's data on the division rate of *Actinosphaerium* gave a temperature coefficient of 2,63, which figure is in close accord with the law of VAN'T HOFF in regard to the velocity of chemical reactions as influenceed by temperature. These workers found that the optimum temperature for *Paramecium aurelia* was between 24° C and 28° C. They then selected animals which were in the same phase of the life cycle, thus making the necessary correction for rhythms. As a result of their experiments they obtained a temperature coefficient of 2,84, and concluded that the rate of fission as influenced by temperature proceeded at a rate similar to that for chemical reactions.

MIDDLETON (1918) while not studying the effect of temperature

on division rate, gives figures which show the effect of temperature on the division rate of *Stylonichia pustulata*. His results are roughly in accord with the data given for MAUPAS for this animal. In one of his initial experiments we find:

60 lines at	30—32° C.	Averaged	2,121	Divisions	per	day	for	1st	ten	days.
„ „ „	18—20 „	„	2,076	„	„	„	„	„	„	„
„ „ „	30—32 „	„	3,018	„	„	„	„	2nd	„	„
„ „ „	18—20 „	„	1,505	„	„	„	„	„	„	„

In a second preliminary experiment he ran 30 lines for 180 days at space temperatures varying from 23° C to 30° C, and 30 lines for 240 days at temperatures varying from 4,5° C to 17° C. Those at high temperatures averaged from 2,330 divisions per day to 3,176 but eventually died out. Those kept at low temperatures started with an initial daily division rate of .780, gradually declined to .076, (ninth 10 day period) then came up to .865 divisions per day (twenty third 10 day period) and were discontinued ten days later with a daily division rate of .655. This is a very marked temperature effect and roughly agrees with the law of VAN'T HOFF.

SPENCER (1924) maintained her cultures of *Paramecium calkensi* in a Freas thermostat at a practically constant temperature (21° —23°) and still got wide fluctuations in the division rate. These she regarded as rhythms, in the sense of WOODRUFF'S definition of the term, for she regarded the environment as constant. Her fluctuations were certainly not caused by temperature.

The isolation cultures of *Paranema* and *Entosiphon* give some additional light on this matter. It was well known that they would both live at low temperatures, for both repeatedly appeared among the organisms found living in water at 4° C to 6° C under several inches of ice covering a pond whose fauna was repeatedly checked by the writer during the winter of 1926—1927. In the laboratory however, one series of *Paranema*, CP, after living for ten months at room temperatures, began a steady decline in the division rate when removed to the refrigerator and died at the end of 110 days. A daughter series, CPA, underwent a similar decline when placed in the refrigerator, and was finally removed to the room to prevent its probable death. This deleterious effect of low temperatures is not wholly conclusive as far as *Peranema* is concerned, for two other series, AP and BP, kept continuously at room temperatures, also died following gradual declines. Series APB and APBA which were kept in the incubator at about 25° C, maintained much higher division rates than those kept at room or refrigerator temperatures,

but APBA was lost by accident while maintaining a high division rate and APB died as did a third incubator series, BPAA. It is evident that room temperatures are nearer the optimum for this animal, for three daughter series of CP were maintaining a fair division rate when the work was discontinued, and a fourth series, APA, showed no signs of a decline in it division rate when it was accidentally lost in July, 1926, after ten months of culture.

The above figures show clearly the effects of low, medium and high temperatures on the division rate of this organism. The terms low, medium and high are of course purely relative, and might not be spplicable to another organism, or even to this organism, under other environmental conditions. The data indicate that division is slowed up markedly at 11°—15° C that it proceeds at a somewhat faster rate around 21° C and that it is accelerated materially at about 25° C. There is some indication that both the high and low temperatures are eventually fatal to this organism, but the data at hand is deemed inadequate for such a conclusion and the matter is under further investigation.

Entosiphon gives more positive indications in regard to temperature. Everytime a culture was subjected to temperatures as low as 11°—15° C there was a decided decrease in the rate of fission. And in every case but one where cultures were subjected to an increased temperature, there was a marked increase in the rate of fission. Table three shows the comparisons of various series in this respect. One series died out in the incubator, but none of the others showed a noticeable decrease while therein. One of the series died out in the refrigerator; in all cases, a series put in the refrigerator showed a steady decrease in the fission rate. There is one noticeable exception; series 2-HEB had a lower fission rate in the incubator than its parents series had at room temperatures.

We may conclude then, that for *Entosiphon*, high, medium, and low temperatures have the same effect on the fission rate as found for *Peranema*, viz, to slow up fission rate; to maintain it at a possible equable rate; and to accelerate it. This is in accord with the various findings of MAUPAS, CALKINS, WOODRUFF and BAITSELL, and MIDDLETON on various infusoria.

The history of the cultures of both these animals which were placed in the refrigerator indicates that temperatures decidedly below the optimum will so slow in the fission rate as to terminate the life of the organism by death. Such an indication is well supported by the histories of series CP and CPA of *Peranema* and EVD,

Table Three.

Series	Where kept	Temp. in ° C	Length of Life	Avg. Div. 10 day Period	Fate
EW	Room	21 (About)	634 Days	14,96	Discontinued.
EVD	Room	21 (About)	40 „	14,75	Transferred.
EVD	Ref.	11—15	120 „	8,25	Transferred.
EVD	Inc.	25	40 „	17,73	Killed.
EVB	Inc.	25	140 „	18,40	Died.
EVC	Inc.	25	150 „	18,07	Transferred.
EVC	Room	21 (About)	170 „	14,41	Discontinued.
2-HE	Room	21 (About)	230 „	14,43	Transferred.
2-HE	Ref.	11—15	120 „	8,50	Transferred.
2-HE	Room	21 (About)	60 „	12,66	Discontinued.
2-HEB	Inc.	25	172 „	14,12	Discontinued.
2-HEA	Ref.	11—15	107 „	7,57	Died.

2-HE and 2-HEA of *Entosiphon*. Woodruff and Baitsell found a similar condition in their experiments to determine the optimum temperatures for the life of *Paramecium aurelia*. Thus at 21° C their cultures lived only 20 days. This weakening of vitality however is probably a laboratory condition, and does not obtain in nature, or is provided for by some mechanism not encountered in laboratory cultures. Furthermore, one series out of three in *Entosiphon* had not died at the end of 120 days in the refrigerator, and longer-lived races are easily possible.

No variations other than those in the fission rate, traceable to environmental influences, have been observed in either of these organisms. Certainly nothing of a heritable nature has been found. In the Infusoria, variation of the metabolic activities has been described as due to youth (Calkins, Woodruff), but Calkins (1926, p. 489) points out that these variations follow conjugation and are lacking in some ciliates, as well as *Actinophrys* and *Eudorina*. Since there is no evidence of youth in *Peranema* and *Entosiphon*, variations in the metabolic activities are not traceable to such a factor.

Middleton (1915) obtained variation in the fission rate of *Stylonichia pustulata* which eventually split a clone into "slow" and "fast" lines, by continually selecting for these differences in the fission rate. Since both types were kept under the same cultural conditions, he concluded that he had a heritable variation due to the slow accumulation of small variations. Calkins states that "in general all results that are based upon physiological differences must be

cautiously interpreted". This is especially applicable to data dealing with the fission rate of bacterial feeders such as *Stylonichia*. Furthermore the validity of MIDDLETON's conclusion is open to doubt if we include his data dealing with the division rates of 60 fast lines of ex-conjugants and 60 slow lines of ex-conjugants. These 120 lines were started within three days after conjugation of the parents, and the number of fissions in each of 5 three-day periods noted. For the first two periods, the "slow" lines gave the most fissions; for the last three periods the "fast" lines gave the most; but the total number of fissions for the "slow" lines was 0,20 per cent greater than for the "fast" lines. This would indicate that his slow and fast variations did not persist through conjugation, although he states they did persist "through and after conjugation". Finally, the average daily division rate of these 120 lines of ex-conjugants was lower in many cases than the average daily division rate of his older lines much further removed from conjugation, which would not indicate any special intensity of metabolic activities due to youth.

Both the *Peranema* and *Entosiphon* cultures have been fairly constant in fission rate when kept under constant conditions. This is borne out by comparing the different series at the different times during the experiment. But a closer comparison may be made within a series. If we take the division rates of the four lines of series EW from October 26, 1926 to December 14, 1926 we find them to be as follows:

	Line (a)	Line (b)	Line (c)	Line (d)
	Divisions in 10 Days			
Oct. 26—Nov. 4	16	17	16	15
Nov. 5—14	14	16	15	18
Nov. 15—24	16	15	17	16
Nov. 25—Dec. 4	16	16	16	16
Dec. 5—14	17	16	16	15
	63	64	64	64

This shows a remarkable uniformity of division rate and while it is perhaps the most uniform for any of the series during as long a period, it is offered as proof that uniparental reproduction offers little variation, and that the genotype is not plastic but well fixed.

c) Lack of Conjugation or Encystment.

There has never been any indication of conjugation or encystment in mass cultures of either *Peranema* or *Entosiphon* as far as the writer could discern. None has ever been recorded as far as available literature has revealed. In mass cultures there are often aggregations of each of these organisms which would lead to a suspicion of conjugation. This is especially true of *Peranema*, for often clusters of individuals are so entangled to make it almost to separate individuals. Pathologic cultures of *Entosiphon* may also present an appearance of conjugation, more deceiving since DOBELL's conjugating cultures of *Copromonas* (1908) were known to be pathologic. But no conjugation has been found, and it has certainly not occurred in any of the isolation series.

Various efforts were made to induce either conjugation or encystment in mass cultures of these animals. Since the causes of these phenomena are not well known or understood, various methods were tried. KATER and BURROUGHS (1926) have summarized the various causes advanced for encystment in protozoa. Among the causes advanced are: Drying up of the medium; starvation; toxic chemical substances in the medium; abundance of food; need for protection during multiple and binary fission; need for quiescent period during which ingested food may be assimilated; hydrogen ion concentration; and necessity for nuclear reorganization.

KATER and BURROUGHS experimented with *Polytomella citri*, a colorless flagellate of the Phytomonadida, and tested the effects of temperature, hydrogen-ion concentration, metabolic by-products and food deficiency. They got encystment under all of these conditions, but nothing conclusive, and finally decided that the factor responsible for it in this organism was the present of abundant starch within the organism. BEERS (1927) finds encystment occurs under a variety of conditions in *Didinium*. It becomes apparent from reading over the literature that there is no one condition which brings on encystment, and that there may be several reasons for its occurrence in one species.

Conjugation also has been investigated without great success as to a determination of its causes. CALKINS (1919) got conjugation after starvation "if the internal conditions of the organism were suitable for it". His general conclusion for ciliates is that conjugation is largely, but not wholly, due to the environment. AUSTIN (1927) thinks the primary cause to be internal, with an external stimulus,

which is perhaps an accumulation of CO_2. ZWEIBAUM (1912) got an indication of salts as a causative factor, but HOPKINS and others have failed to get the same results, with the methods of ZWEIBAUM, and working on the same organism, *Paramecium caudatum*. HOPKINS (1921) got an augmentation of intensity of conjugation but no initiation of it. His final conclusion is that it appears to be initiated by a period of unregulated division. BAITSELL (1912) found that on beef extract, *Stylonichia pustulata* would conjugate, while on hay infusion he could get no conjugation, and believes that the latter environment was unfavorable. He attributes conjugation to external factors. His conjugations in beef broth were infertile however and he thinks neither medium was fully suitable for *Stylonichia*. HANCE (1917) believes that conjugation in *Paramecium* is caused by the concentration of katabolic products in the culture medium. He got this concentration mainly by rapid evaporation of the medium, or in dense mass cultures. Again we note a lack of agreement as to the causes of conjugation. CALKINS has undeniably shown that it satisfied a protoplasmic need in *Uroleptus mohlis*, but other protozoa apparently can live indefinitely without it, despite the fact that it may be a common occurrence in their existence, as in DAWSON'S long continued culture of *Blepharisma*, or HARTMANN'S long-lived cultures of *Eudorina*.

Many efforts were made to induce either conjugation or encystment, or both for *Peranema* and *Entosiphon*. Starvation as used by MAUPAS never gave any results with either few or many organisms in a dish. Quick and slow drying up, both with abundant food and with little food, proved futile. One culture left without food in a moist chamber still had a few animals in it after four months. Freezing or partial freezing, and high temperatures as well had no effect other than to kill the animals if carried to extremes. Changing from one medium to another was repeatedly tried and gave no results other than to show that the animals would live or even thrive on several other media than the cracked wheat. Various concentrations of NaCl, KOH, $NaHCO_3$, the three potassium phosphates, magnesium sulphate, sodium nitrite and nitrate, ammonium molybdate, a number of the amino acids as leucine, alanine, asparagin, histidine, and a number of the organic acids as oxalic, phosphoric, tartaric and citric, were tried. Several ranges of p^H were tried, coincident with trying the above chemicals and some toleration of p^H range was found, but no encystment or conjugatien. Cultures were carried in Novy jars at reduced atmospheric pressures, and in

a few instances stood the treatment well. CO_2 was also substituted in these jars, and H_2S but nothing came of these attempts.

The above attempts have embraced practically all of the methods wich have been used elsewhere. Once, in 1923, a doubtful case of encystment occurred in a mass culture of *Entosiphon* living in the synthetic medium of PETERS. No amount of repetition has since produced it, and it is more probable that it was a pathologic case.

Encystment then, is a remote possibility for *Entosiphon*, and there is not the slightest indication of it for *Peranema*. Conjugation may possibly occur, but there is no indication whatever that it does, in either of these organisms.

These organisms were examined cytologically hundreds of times in the course of this work. No reorganization processes other than those described above for *Entosiphon*, and by other workers (HARTMANN and CHAGAS, 1910; HALL, 1928) for *Peranema*, have been found. The synkaryon formation of conjugation has been totally lacking in our cultures. Nothing analogous to the processes of endomixis in ciliates has been found. The reorganization processes of cell division are orderly and follow a certain course with no visible sign of deviation, generation after generation. Division processes seem to be regulated and leave the protoplasm reorganized, labile. This reorganization is shown in *Entosiphon* by the nuclear changes, by the absorption of the old siphon and its replacement by new ones, by the appearance of new flagella and new basal granules for one of the daughter organisms. The reorganization takes the place of amphimixis or endomixis, and these are therefore unnecessary, placing *Peranema* and *Entosiphon* in the same category with *Eudorina*, *Actinophrys* and *Paramecium Calkensi*. The reorganization processes are able to maintain the vitality of the animals at a normal, constant level and hence continuous agamic reproduction is sufficient for the maintenance of the race in these animals.

Summary and conclusions.

1. *Entosiphon sulcatum*, a colorless, saprophytic member of the Euglenida has been carried in isolation culture continuously for 634 days, without conjugation or endomixis during 947 generations, and with no sign of waning vitality.

2. *Peranema trichophorum*, a colorless holozoic member of the Euglenida has been carried in isolation culture continuously for 420 days, without conjugation or endomixis during 171 generations, and with no evidence of waning vitality.

3. It has not been possible, experimentally or otherwise, to produce conjugation or encystment in cultures of these organisms during this time.

4. There is no evidence of rhythms in the life cycle of these organisms. Fluctuations in the division rate are due to environmental conditions. The organisms tend to maintain a constant level of vitality under a constant environment.

5. Temperature directly affects the division rates. At 25° C the division rate is high but cultures tend to die out suddenly; below 15° C the division rate is slow and gradually declines until, in the case of *Entosiphon*, death ensues. The optimum temperature at which the division rate is maintained under laboratory conditions is near 20° C.

6. The temperature coefficient for the rate of fission in *Entosiphon* is about 2,00 which is in agreement with the law of VAN'THOFF.

7. Neither amphimixis nor endomixis is necessary for the continued life of these organisms.

8. Continuous agamic reproduction with the reorganization processes of cell division already described, is sufficient to maintain the race.

9. No evidence of any variation has been found, thus supporting the statement that the genotype remains fixed under uniparental inheritance.

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