THE CAUSE AND NATURE OF ENCYSTMENT IN *POLYTOMELLA CITRI.*

J. McA. KATER AND R. D. BURROUGHS,

PRINCETON UNIVERSITY.

Although encystment has long been known to play an important part in the life of most protozoa the cause and nature of the phenomenon has been the subject of relatively little careful experimental work. The evidence derived from the earlier work, slight as it was, together with the fact that cysts of protozoa are very resistant to drying, toxic substances, etc., has led the writers of text-books to speak of encystment as a passive response to adverse environmental conditions (Calkins 1909, Minchin 1912, Doflein 1916). In spite of the fact that such generalizations are quite common, there is little evidence that, on critical examination, cannot be interpreted otherwise than as mentioned above. The general confusion that has arisen from the conflicting data of almost every piece of work on this subject makes it desirable to give a brief historical review.

From the time that encysted protozoa were first observed in 1769 by Saussure until 1855 the few observations made on this subject did not deal at all with the cause of the process, but merely with establishing the relationship between cysts and the active forms to which they give rise. In 1855 Cienkowsky not only called attention to the fact that encystment is probably not universal among protozoa (as was supposed before that time) but also succeeded in producing encystment by a lengthy drying up of the medium.

After this date dessication was considered to be the principal, if not the sole cause of encystment, until the subject was attacked by Maupas and Fabre-Doumergue in 1888. The former produced encystment in predaceous ciliates by the deprivation of food, while the latter showed that drying up of the medium was not of paramount importance. By growing several ciliates in cultures placed in a moist chamber, he discovered that they

regularly encysted. This author attributed the causal factors to putrefaction of the medium, resulting in the release of toxic chemical substances.

The contention of Maupas has received support from other workers. Among them Root (1914) concludes that lack of food is the cause of encystment in the suctorian Podophrya collini. Mast (1917) obtained similar results with *Didinium nasutum*. He says: "Encystment in Didinia can usually be induced by cutting off the food supply. But it frequently occurs when there is an abundance of food present and sometimes it does not occur when there is none." However, Mast and Ibara (1923) after some carefully conducted experiments decide that "They encyst more freely in cultures supplied with food than in those without food, and this probably is due to greater increase in numbers, resulting in greater accumulation of waste material in the one than in the other." Hogue (1915) observed that the limax amœba upon which she was working encysted in the presence of abundant bacteria, upon which it feeds, and that the cell body of the encysting amœba always contained numerous bacteria. In both this paper and a later one (1917) she expresses the opinion that accumulation of byproducts of metabolism and oxygen deficiency are the principal causal factors. She thinks that they operate by causing the amœbæ to lose the power of assimilation. Carter (1919) concludes that abundant food is necessary for encystment of *Amœba proteus*.

Kofoid and Swezy (1921) consider that encystment in the marine dinoflagellates is due to one of two causes, the need for protection during the period of multiple and binary fission, and the need for a quiescent period for the assimilation of a large food body that has been ingested. Hall (1925) assigns *Oxyrrhis marina* to the second group given by Kofoid and Swezy. In addition, referring to drop cultures sealed with vaseline, he says: "After six weeks, exclusively non-motile forms were observed; in these, no flagella could be detected in observation under the oil immersion objective, and in some cases a 'cyst wall' seemed to surround the flagellates." Since no food vacuoles were present, he thinks that these non-motile forms may represent cysts formed in response to unfavorable conditions. From his

figures of cysts one would think that there should be no question of the presence of a cyst wall if one were there and it seems doubtful that they were really cysts.

Stolte (1922) reports that the ciliate *Blepharisma* encysts only in the presence of abundant food, and that large food vacuoles are present in those entering encystment. He also emphasizes the necessity for a relatively large number of *Blepharisma* in the culture before encystment is possible. This conclusion concerning a ciliate is quite in harmony with Kofoid and Swezy's for dinoflagellates.

Koffman (1924) suggests hydrogen-ion concentration as one of the essential factors in producing encystment in ciliates. He says: "The ciliates require ordinarily a definite P_h zone with a definite optimum, for their development. Below the minimum as well as above the maximum either initiates an encystment or the animals are destroyed."

In direct contradiction to the conclusions of the two preceding authors, we might mention the work of Brand (1923) who considers that, in the case of *Vorticella microstoma*, hunger, oxygen deficiency, and gradual drying up are the principal external factors for encystment. He relegates products of metabolism and chemical influence in general to a position of less importance.

Calkins (1915) found that *Didinium nasutum* regularly encysts entirely independent of adverse environmental conditions. However, encystment in this ciliate may not be entirely comparable to that of other forms, as a nuclear reorganization, similar to endomixis, occurs during the encysted phase. Fermor (1913) has described the same sort of reorganization in *Stylonychia pustulata*. With regard to the significance of encystment in *Didinium* Calkins says: "In addition to the casual encystment resulting from adverse environmental conditions, there is another form of encystment which involves more deep lying activities of the protoplasm," and again, "When the process is completed and the organisms emerge from their cysts they possess from five to seven times the vitality, measured by the division rate of the same race prior to encystment." In the face of this statement it is interesting to quote Mast (1917): "there was no evidence obtained indicating that conjugation or encystment has any

effect on death rate, fission rate, or variation in fission rate. This would indicate that neither of these processes is a rejuvenating process, at least not in the sense in which Calkins has used the term."

The foregoing review certainly indicates that more experimental work is needed on this subject, not only to obtain a better understanding of the protozoan life cycle, but also for the elucidation of the general biology of the so-called "resting forms of protoplasm." With this in view we have undertaken the work recorded in the following pages. The colorless phytomonad, *Polytomella citri* was selected as the organism to be experimented upon for two reasons: (*a*) it encysts very readily, and (*b*) most of the previous work has been confined to ciliates and amœbæ and we thought it desirable to extend the experimental field to flagellates.

We wish to express our gratitude to Professor E. G. Conklin, not only for his criticism, but also for his interest and encouragement. Our thanks are also due to Professor E. Newton Harvey for some very valuable suggestions.

Material and Methods.

The culture medium used in all of the experiments described in the following pages was made by boiling timothy hay in distilled water, about ten grams of hay to a liter of water. Three liters was the quantity usually made at one time, and all of the cultures of any one experiment contained medium of a single making, thus eliminating the possible error due to variation in the medium. Test tubes served the purpose of containers. Approximately 15 cc. was the quantity of medium in each culture.

Attempts were made to determine the effect of (1) various temperatures, (2) hydrogen-ion concentrations, (3) metabolic by-products, and (4) food deficiency on the tendency to encyst.

(1) The different temperatures were chosen because of their availability; and in spite of the fact that there was considerable variation in some cases the results were sufficiently striking for the present purpose. No attempt was made to arrive at an optimum temperature within a narrow range, as this was not thought to be necessary.

(2) For the work on hydrogen-ion concentration the ordinary hay infusion was used and the P_h was altered by either sodium hydroxide or acetic acid. The hydrogen-ion concentration was determined by the colorimetric method.

(3) The problem of metabolic by-products was approached in two different ways: first, by filtering the cultures through a porcelain filter and replacing the medium with fresh hay infusion, and, second, by removing the fluid from a thriving culture, in the same way, and replacing with medium taken from an old culture in which there were none active, all being encysted. In the one case the by-products were kept at a minimum, in the other they were increased beyond normal.

(4) To obtain a low food content in the cultures the hay infusion was replaced with distilled water and various percentages of distilled water and fresh hay infusion.

It has become rather customary, in recent years, to carry on work of this kind on organisms that have all been derived from a single individual. By this method one avoids comparing different strains, but, on the other hand, the results are applicable only to that particular strain, which probably can never be duplicated. The organisms used in the present work were all derived from a single culture, not from a single individual. It was thought, at the beginning, that by running a great number of cultures conclusions could be arrived at that would be applicable to the species, and that minor variations could be smoothed out by amount of data. The remarkable correspondence in the different cultures of any one series has entirely justified this procedure. This agreement has been so great that it has been possible to give only one result for all cultures of a series, with only two exceptions.

The condition of the cultures, in respect to active forms, is designated by the following terms: *very poor*, *poor*, *fair*, *good*, and *excellent;* and, with regard to cysts by: *very few*, *few*, *some*, *many*, and *very many*. These indefinite terms will doubtless arouse criticism. To this we can only reply that the differences in both active and encysted forms in the different series was so striking in all cases that they could easily be placed in their proper class, and, secondly, that it is the relationship which is of importance and not the actual numbers.

Polytomella was associated with bacteria and yeast in all of the cultures.

Experimental.

Temperature.—The procedure determined upon was to eliminate temperature first and then carry on the remainder of the experiments at the optimum temperature. Accordingly, thirty-six cultures were seeded and twelve of these were kept at each of the following temperatures: 10°–11° C. (series *A* 1), 21°–22° C. (series *A* 2), and 22°–27° C. (series *A* 3). Series *A* 3 was kept in the laboratory, which explains the wide variation in temperature. At the end of nine days all cultures of the three series were in excellent condition. Those of series *A* 1 showed no cysts, *A* 2 contained few, while some were found in *A* 3. In fourteen days *A* 1 was in good condition with no cysts; *A* 2 was in fair condition, with some cysts; and *A* 3 was poor, with many cysts. In twenty-one days *A* 1 was only fair and no cysts had yet appeared; *A* 2 had reached the same condition that *A* 3 was in on the fourteenth day and *A* 3 now contained no active forms and many cysts. The active forms had entirely disappeared from *A* 2 by the thirty-fourth day. These two series continued in the same condition for the remainder of the experiment. On the thirty-fourth day *A* 1 was in poor condition, without encystment. From the thirty-sixth to thirty-ninth day there was no ice in the refrigerator and the temperature rose from 10° to 21° C. At the end of those three days all twelve cultures were in fair condition and a few cysts had appeared in five of them. On the thirty-ninth day ice was again placed in the refrigerator and the experiment continued for twenty-one more days. At the end of that time there were practically no active forms in any of the cultures and the few cysts that were in five of the cultures on the thirty-ninth day were still present, but the remaining seven cultures had died out without any encystment whatever.

The first attempt showed that the cultures kept at 35° C. would not develop. Consequently, to attempt to determine the effect of so high a temperature, cultures were permitted to develop in the laboratory for a few days and were then placed in the incubator. It was found that at 35° thriving cultures would gradually die out until, at the end of two days, they would have com-

pletely disappeared without the formation of a single cyst. A little later twelve cultures were seeded and six of these were placed in the laboratory, the other six at a temperature of 30° C. In two days all were excellent cultures and many cysts had appeared in the ones kept in the laboratory, while none had encysted at 30°. On the third day all were alike, fair cultures with many cysts. The reason for these encysting more quickly than those of Series *A* will be explained later.

A second set of temperature experiments was started some time after series *A*. Series *B*1, *B*3 and *B*4 were kept at the same temperatures respectively as *A*1, *A*2 and *A*3. Series *B*2 was kept at 15°–22° C. With only slight variation the results were the same in series *B* as in *A*. Observations were made earlier than in *A* and it was found that the cultures kept at the lower temperatures, developed much more slowly than at the higher ones, such as laboratory temperature. Series *B*1 and *B*2 were still in rather poor condition on the seventh day while *B*4 was in good condition and a few cysts were present.

As noted above, a very few cysts appeared in five of the cultures of *A*1. This was not true for *B*1 as no encystment whatever occurred. The experiment was discontinued on the fifty-first day and seven cultures of series *B*1 had completely died out, the remaining five were in fair condition. These five were permitted to remain in the laboratory and in five days they had completely died out without any encystment.

We see that, as a whole, no encystment occurs at extremely low or high temperatures. Behavior at high temperatures differs from that at low in that, in going up the scale, encystment is not prevented until a point is reached which will kill the flagellates in a few days, while at low temperatures they develop more slowly and make very long lived and good cultures.

How are these results to be interpreted? At low temperatures one would expect the rate of metabolism to be lower and the time before encystment to be longer, but when the cultures died out without encystment a new light was thrown on the problem. If encystment were a response to adversely low temperatures we would expect it to occur there first. On the other hand, if encystment were a response to adversely high temperature we

would think that the two days which the cultures lasted at 35° C. would have been sufficient for the organisms to encyst rather than die. It is also noteworthy that the six cultures at 30° did not encyst until after their controls. It seems entirely justifiable to conclude that extremely low or high temperatures prevent encystment and that medium temperatures are not only conducive to rapid growth and division but also to encystment.

Hydrogen-ion Concentration.—It was noted in the above account that room temperature was the best one employed for the growth and division and encystment of *Polytomella citri*. Consequently, all of the remaining experiments were carried out at room temperature.

The procedure for the work on hydrogen-ion concentration has largely been given. Unaltered hay infusion (P_h 5.4) was used for the controls and, of course, these were not altered for the duration of the experiment. For the remaining cultures the P_h was restored to that at which the cultures were started on the third, ninth, and sixteenth days. Forty-eight cultures were seeded and the hydrogen-ion concentration was changed to the following P_h values (six cultures in each series): 3.5 (series *C*1), 4.5 (series *C*2), 5.4 (series *C*3), 6.2 (series *C*4), 7.5 (series *C*5), and 8.5 (series *C*6). Twelve cultures were kept as controls (series *C*7).

By the third day the cultures of series *C*1 were in fair condition and so they remained for the first week. After that the numbers decreased to such an extent that all could truly be classed as poor cultures. On the twenty-second day they were still poor and no cysts had been formed. Series *C*2, *C*3, *C*4, and *C*5, were practically identical throughout the first twenty-two days. This correspondence was so great that it is unnecessary to give separate results for each, which would be merely repetition. They were in good condition on the third day and excellent on the fifth. A very few cysts appeared on the fifth day. On the twenty-second day, the cultures were still in good condition and some cysts were present. Series *C*6 remained rather poor throughout the experiment and only a very few cysts were found on the twenty-second day.

The controls (*C*7) behaved quite differently from the other cultures. On the fifth day all twelve cultures of *C*7 were in

excellent condition and some cysts were present. By the eleventh day many cysts were found, while in the other series this condition was never reached. The cultures were only fair on the twenty-second day and very many cysts were present.

We now see that the cultures did excellently at any P_h from 4.5 to 7.5 and that encystment occurred with equal facility at any point within that range. However, in no experimental case was encystment as great as in the controls.

This experiment has been followed only to the twenty-second day, but observation was not discontinued at that time. They were examined on several occasions, but it will suffice to give only one of these, that made on the thirty-fourth day. All cultures were rather poor, so far as active forms are concerned and many cysts were present, though not as many as were found in the controls. This data, together with some which will follow, indicates that it was not the actual hydrogen-ion concentration which prevented the experimental cultures from equaling the controls in rapidity of encystment, but the sudden changing of the medium when the P_h was altered.

These results are quite contradictory to those of Koffman, and are more in harmony with Brand's conclusion that chemical influences are of little importance in producing encystment.

By-Products of Metabolism and Food Supply.

These two are taken up together partly because similar methods were used in dealing with them and partly because both factors entered into some of the experiments.

An attempt was first made to determine the effect of removing the by-products of metabolism by filtering the cultures through a porcelain filter and replacing the medium with fresh hay infusion. Twenty-four cultures were seeded and six of these had their culture medium replaced with fresh hay infusion every day (series *D*1) six others every second day (series *D*2), six others every third day (series *D*3) and the remaining six were kept as controls (series *D*4). The medium was not replaced in any of the cultures after the tenth day.

The results of this work are very startling. Whereas in all previous experiments the controls behaved beautifully, encysting

in about a week, the controls in this case did not encyst until the tenth day and then very few cysts appeared, most of the active forms dying rather than encysting. Although all the cultures of *D*4 (controls) were in excellent condition on the eighth day they had entirely died out by the fifteenth day and few cysts were present. The cultures of series *D*2 and *D*3 did not die out as soon as those of *D*4 nor did they encyst as quickly. On the twenty-second day of the experiment they had reached the same condition in which *D*4 was found on the fifteenth. By the thirtieth day all active forms had disappeared from the cultures of *D*1 and slight encystment had occurred.

Not knowing what to make of this behavior, twenty-four more cultures were seeded and the experiment repeated. These cultures were divided into four series *E*1, *E*2, *E*3, and *E*4, each series corresponding to the similarly numbered series of *D*. This yielded identically the same results as *D* with the exception that *E*1 and *E*2 died out without any encystment whatever.

It is well to state here that *D* was seeded from a culture of *C*1 shortly before it completely died out and *E* was seeded from a culture of *C*2 after it had reached a very poor condition. No cysts were transferred, only active individuals. Series *C* had likewise been seeded from *A*3. In an attempt to find the explanation for this loss of ability to encyst on the part of series *D* and *E*, some cysts were taken from *C*6, placed in fresh hay infusion and permitted to excyst. On the next day eighteen cultures were seeded from these newly excysted ones and two days later it was found that every one of the eighteen contained many cysts. As a check on this, cysts were taken from *C*7, washed in seventy per cent. alcohol, and permitted to excyst in fresh hay infusion. From these, thirty-six cultures were seeded and on the second day they were found to have repeated the activity of the eighteen, being largely encysted. In addition, cysts were taken from *E*3, washed in alcohol, excysted, and twelve cultures seeded from them. At the end of two days many cysts were found in all of the cultures.

By putting the last two paragraphs together, one gets a strong indication that the longer *Polytomella citri* is removed from encystment, the less is their tendency to encyst.

To carry this further, series *F* was started. *F*1 (six cultures) was seeded from *A*1 on the sixtieth day after that series was seeded; *F*2 (six cultures) was seeded from *E*2 on the eighteenth day; *F*3 (six cultures) was seeded from active ones that were ten days removed from newly encysted individuals; and *F*4 (eighteen cultures) was seeded from active ones excysted only five days previously.

On the third day all cultures were excellent, with the exception of *F*1, which were in fair condition. No cysts had appeared in the first three series, but some were present in the cultures of *F*4. By the seventh day *F*1 and *F*2 were in excellent condition, with no cysts while the cultures of *F*3 were in good condition with some cysts and *F*4 was only fair with many cysts. Examination on the nineteenth day revealed the fact that the cultures of all four series were very poor. A very few cysts had appeared in several of the cultures of *F*1; there were none at all in *F*2 while *F*3 and *F*4 contained many cysts.

It is now clearly seen that the longer encystment is prevented, whether by low temperature (*F*1) or by long continued transfer (*F*2), the less is the tendency to encyst, and if this procedure be sufficiently extended the ability to encyst is entirely lost. The morphological basis for this is given in the next section.

It was determined to repeat the work of *D* and *E*, using newly excysted forms. Accordingly twelve cultures were seeded and six of these had their medium replaced every day, the others were kept as controls. In two days the controls were quite generally encysted while those filtered every day showed no cysts until the third day.

Some cultures of the types found in *F*2 and *F*4 were filtered and the medium replaced with fluid taken from cultures that had entirely encysted. In the former no encystment whatever occurred, while in the latter it was slowed up, usually for about one day, as compared to the controls. When first placed in such a medium, we were surprised to find that division was apparently stimulated, as examination under the microscope would reveal a great many late division stages, which are the only ones that can be detected in living material. Although encystment was slightly deferred in the case of those of type *F*4, the cultures

themselves in all instances ceased to show any active forms before they disappeared in the controls. This was probably due to the lack of food.

Now let us inquire why *E*1 and *E*2 did not encyst at all. Was it because of the removal of the by-products of metabolism? The evidence is very much against such a conclusion. If that were true, we should have expected the cultures of the last mentioned experiment to have encysted before the controls, since in that case metabolic by-products were probably increased. Also, in the case of the cultures seeded with newly excysted individuals in which the medium was replaced with fresh hay infusion every day, we should have expected encystment to have been postponed more than it was, if metabolic by-products were an important factor for encystment. Again in series *E*1 and *E*2 active forms remained in the cultures for ten days after filtering was stopped. This, we think, would have been sufficient time for the necessary by-products to accumulate, yet no encystment occurred. The arguments just recounted seem to entirely justify the conclusion that the filtering of a culture and replacing its medium with fresh hay infusion defers encystment, but this effect is produced by the stimulus to growth and division given the organisms by changing the medium suddenly, and is not due to the removal of an adverse environmental factor created by the presence of metabolic by-products. This is quite comparable to seeding a new culture.

The reader will doubtless have thought of the fact that an ever plentiful supply of food was present during the early course of *D*1, *D*2, *D*3, *E*1, *E*2, and *E*3. However, the same arguments that applied to metabolic by-products will apply here and we can likewise conclude that food deficiency is not the cause of encystment. This was carried further by filtering thriving cultures and replacing their medium with distilled water, 25 per cent. hay infusion and 75 per cent. distilled water, etc. to pure hay infusion, a change of 25 per cent. in each step. In the cultures that received pure distilled water no encystment took place and the cultures died out in two days. In the others, however, neither fission nor encystment seemed to be materially affected.

A few experiments were carried out which may have involved

oxygen deficiency. Thriving cultures of Polytomella were sealed in glass tubes, very little air space being left between the top of the medium and the sealed end. Since no photosynthetic organisms were present, it would seem as though the dissolved oxygen of the medium should have decreased. At the end of one week the cultures had died out and very few cysts had been formed.

Morphological Changes Accompanying the Experiments.

When a hay infusion culture is seeded with *Polytomella citri* taken from a stock culture (one containing uncooked hay in distilled water) the organisms will multiply rapidly for a few days, during which time staining with iodine will indicate the presence

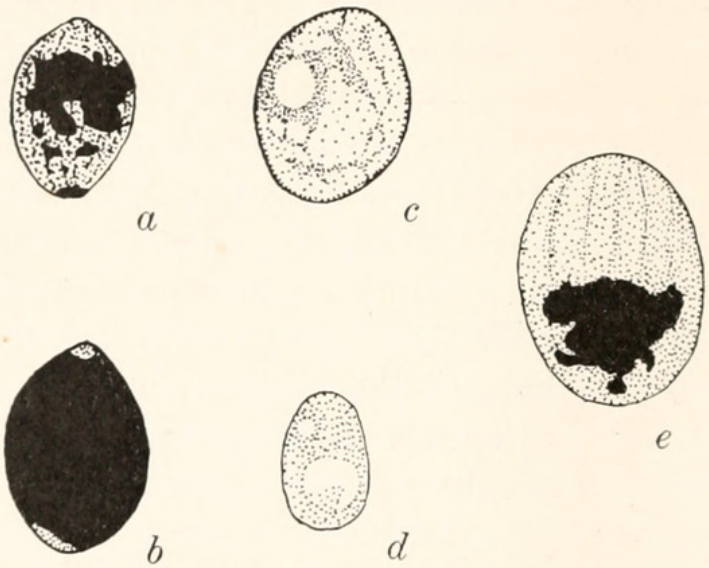

Fig. 1. Diagrammatic figures traced from photomicrographs of Polytomellas stained with iodine. Solid black indicates starch. Equal magnification. (See text for further explanation.)

of relatively little starch (Fig. 1*a*). At the end of from two to five days it will be noticed that the starch increases to such an extent that the entire cell seems filled with it (Fig. 1*b*). It is at this time that encystment occurs. Polytomella never encysts except when the cell-body is filled with starch, and, on the other hand, such a culture has never been found to be unaccompanied by cysts.

As stated above, all cultures used in this work were derived from a single stock, and later cultures were seeded with active ones taken from previous experiments.

It was not only found that when *Polytomella* is so transferred from culture to culture for some time their tendency to encyst becomes practically negligible, but also that marked morphological changes occur. When encystment is thus prevented for some time the organism will frequently show considerable decrease in size, almost invariably the cytoplasm will become coarsely vacuolated, and the starch entirely disappear. Fig. 1*c* was taken from series *E*4 on the fourteenth day after seeding; Fig. 1*d* from series *A*1 on the sixtieth day. In series *D*1, *D*2, *D*3, *E*1, *E*2, and *E*3, it was noticed that all contained little starch. In living mounts the cell-body was quite clear and the size above normal. Fig. 1*e* was taken from *E*2 on the eighteenth day. The abnormally large size was lost as soon as the numbers began to decline. Eventually all of these cultures showed only a few degenerate, vacuolated individuals.

It was thought desirable to determine whether or not these degenerate forms could give rise to healthy cultures. Accordingly, series *F* was started. The history of these cultures has already been given, with the exception of the data found in Fig. 2. Fig. 2*a* was made from a typical individual taken from

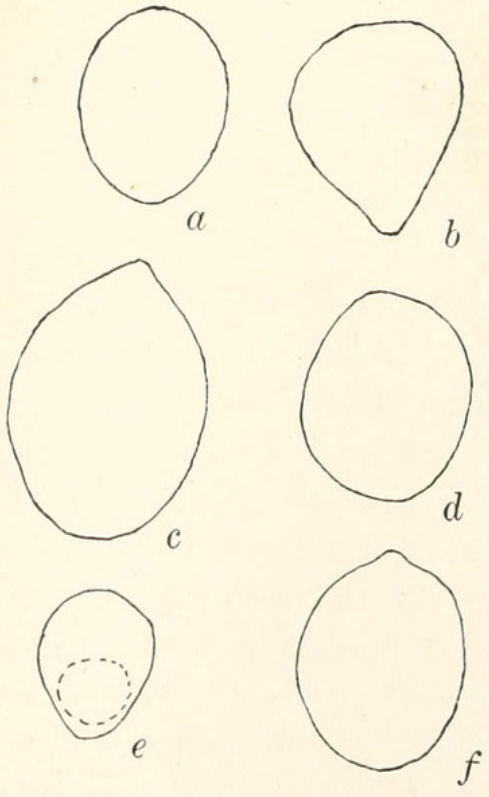

FIG. 2. Camera-lucida outlines showing variations in size. Figure e vacuolated. (See text for explanation.) Magnification 1825 ×.

the culture which furnished the starting point for *F*4; Fig. 2*b* was taken from *F*4 two days later. Fig. 2*c* was taken from *E*2 on the day that *F*2 was seeded; 2*d* was taken from *F*2 two days later. Fig. 2*e* was taken from *A* 1 on the day that *F*1 was seeded; Fig. 2*f* from *F*1 on the second day. A glance at these figures will show that transference of these degenerate forms to a new and favorable medium results in a return towards normality, with the exception of the formation of starch, as *F*1 and *F*2 formed little of that material.

Discussion.

The Causes of Encystment.—In the preceding section attention was called to the fact that *Polytomella citri never encysts except when the cell body contains abundant starch.* Since this is so it seems as though the search for the external factors for encystment becomes resolved into a search for the factors which encourage the formation and storage of starch. Adverse conditions of the environing medium certainly would not be conducive to the formation of this material, at least the above experiments so indicate, and, consequently, we need not be surprised at the conclusion drawn from the present work, namely, *Polytomella citri* encysts only under such conditions as are favorable for growth and reproduction, and adverse conditions lead to death of the culture rather than encystment. This very thing, however, may prolong the life of a culture, so far as active forms are concerned.

The presence of abundant starch in *Polytomella* as a prerequisite for encystment is quite in harmony with the observations of Hogue on *Amœba limax*, Carter on *Amœba proteus*, Kofoid and Swezy on marine dinoflagellates, Stolte on *Blepharisma*, Mast and Ibara on *Didinium*, and Hall on *Oxyrrhis marina*. In fact, most of the recent work indicates that abundant food is much more conducive to encystment than is starvation, which was formerly supposed to be one of the principal causes of encystment, and is still so given in text-books.

In the introduction a quotation was given from Koffman (1924). From this it is seen that altering the P_h to a lethal point either resulted in death or encystment, generally death.

From this he concludes that hydrogen-ion concentration is probably one of the most important factors in producing encystment. It seems to us that he would have been justified in concluding that adverse hydrogen-ion concentration might slightly speed up the process, if the internal condition of the protozoan disposed it towards encystment. It is just such conclusions as Koffman's that have led to an over-emphasis of the importance of adverse environmental factors in producing encystment and have led to some authors speaking of the phenomenon as a "passive adaptation to adverse environment." Although that may be true in a few instances, a critical review of the literature certainly does not indicate that it is the general rule.

The Nature of Encystment.—In a former paper Kater has shown that extensive changes occur during the encysted stage of *Polytomella citri*. This does not particularly involve the nucleus, the budding off of the new centriole and basal granule from the karyosome being the only possible regenerative change that was found. The most striking changes take place in the cytoplasm. The starch rapidly disappears when this resting phase is entered, and, coincident with its disappearance, the metachromatic granules are formed in great abundance. These are absorbed before excystment occurs.

Before attempting a discussion of the meaning of encystment in this flagellate, we must decide why prevention of encystment for several months results in loss of the tendency to store starch, and, consequently, to encyst. On a priori grounds we can say that it is either due to selection, direct effect of the environment, or a combination of the two. Although we may not be able to answer this conclusively, the present evidence indicates that selection does not enter into the matter. If it were due to selection of an encysting strain we should not expect cysts taken from $E3$ and $C7$ to give rise to cultures that would encyst in exactly the same time, two days, yet such is the case. $C7$ encysted much more quickly than did $E3$ and, if there were anything in the strain, the offspring should behave likewise. In the second place, $F1$ showed only a very slight tendency to encyst, mostly dying. Since these cultures were seeded from

*A*1 on the sixtieth day we must suppose, if it is a matter of selection, that the non-encysting variations are more viable than the encysting. This does not seem reasonable, because if it were so we would hardly expect any existing *Polytomella* to exhibit the phenomenon.

No attempt has been made to accurately measure the division rate in a non-encysting strain, such as *F*2, as compared to an encysting, *F*4. Mass observations lead us to believe that even when *Polytomella* has reached a condition of morphological degeneracy and may have entirely lost the power to encyst when they are transferred to a fresh and suitable medium, they are stimulated to a perfectly normal period of growth and division, with the exception that it does not end in encystment.

The morphological degeneration observed in the cultures *A*1, *B*1, *D*, *E*, etc., is very similar to that noted by Maupas in *Stylonychia* when conjugation was prevented and by Calkins in *Uroleptus*. We will not enter into the propriety of terming this senescence, and encystment as a rejuvenating process.

Summary.

1. *Polytomella citri* encysts only when the cell-body contains considerable starch.

2. Encystment is not due to any perceptibly adverse environmental factors.

3. Optimum conditions for growth and reproduction are concomitant with maximum encystment.

4. Prevention of encystment, either by continuous transfer or by low temperature, if carried to sufficient extent, will result in morphological degeneracy and loss of the tendency to store starch and to encyst.

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