A study of encystment in the ciliate, *Woodruffia metabolica.*

By

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With 2 figures in the text.

Introduction.

For the past two years a clone of *Woodruffia metabolica* has been maintained in this laboratory without any signs of senility and degeneration appearing in the clone. During this time individuals have been transferred daily into large depression culture dishes with a supply of *Paramecium multimicronucleata* for food. Throughout this period of time the reproductive rate of the *Woodruffia* has averaged between $2^1/_2$ and 3 fissions daily and, in all, about 2000 generations have been followed. Conjugation has never been observed. From time to time the number of cultures started daily has varied from five to twenty. Often when some of the cultures were discontinued they were left in the moist chambers for a day to several days. Examinations of these discontinued cultures have shown in every instance the beginning of encystment with the disappearance of the *paramecia* and 100 per cent encystment in from 6 to 12 hours after all the *paramecia* had been eaten. In our experience with these cultures no formation of permanent resting cysts occurred, except in a few sporadic cases to be discussed later, until there was an absence of food in the cultures.

Beers (1928 and 1930) has found, in extensive experiments with the carnivorous ciliate, *Didinium nasutum* which also feeds on *Paramecium*, that the chief factor which brings about encystment is the

absence or scarcity of food. CALKINS (1915) found that *Didinium*, when fed on a restricted number of *paramecia*, showed a gradual lowering of the fission rate and an increase in the percentage of encystment until, by the end of the fifty-fifth day, 100 per cent encystment occurred. MAST (1917) carried *Didinium* over 1000 generations without a decrease in reproductive rate and with the absence of encystment. MAST, however, provided an excess of food for the didinia at all times. BEERS was able to show in his experiments that he could reproduce both the results published by CALKINS and those published by MAST, depending upon whether he fed the didinia on a restricted number of *paramecia* or furnished them with *paramecia* in excess. Our results with *Woodruffia* are comparable to those of BEERS with *Didinium*, in that the *Woodruffia* normally will not form resting cysts as long as food is present in excess. When the food supply is depleted 100 per cent encystment occurs in a very short time. In this respect *Woodruffia* differs from *Didinium*. There is no prolonged gradual lowering of the fission rate and a gradual increase in encystment over several days; encystment sets in abruptly and the period of encystment in a culture is short.

Several other workers have found that lack or scarcity of food causes encystment in a number of different protozoa. Studies by MAUPAS (1888) on certain predacious ciliates, by ENRIQUES (1907) on *Vorticella*, by FERMOR (1913) on *Stylonychia*, by ROOT (1914) on *Podophrya*, by MOORE (1924) on *Spathidium*, by ADOLPH (1929) on *Colpoda*, by WEYER (1930) on *Gastrostyla*, by PENN (1935) on *Pleurotricha* and by TAYLOR and STRICKLAND (1938) on *Colpoda*, all show that lack of food is a very important factor in inducing encystment in these different forms.

On the other hand several investigators have found that an abundance of food is necessary for encystment in certain organisms. The results of HOGUE (1915) on *Vahlkampfia*, CARTER (1919) on *Amoeba*, KOFOID and SWEZY (1921) on marine dinoflagellates, MAST and IBARA (1923) on *Didinium*, KEPNER (1924) on *Vampyrella*, STOLTE (1924) on *Blepharisma* and KATER and BURROUGHS (1926) on *Polytomella* show this to be the case.

In addition to the food factor, both the presence and absence of it, as a cause of encystment, still other environmental factors have been reported as causes of encystment in some of the protozoans mentioned above, and in others. CIENKOWSKI (1855), working with a number of protozoans, and GARNJOBST (1937), working with *Stylonethes sterkii* and *Euplotes taylori* were able to induce encystment

by gradual evaporation of the culture medium. CLEVELAND and SANDERS (1930), using *Entamoeba histolytica* and BARKER and TAYLOR (1931), using *Colpoda cucullus* found that crowding of the organisms was essential for encystment. The induction of encystment in *Colpoda* with changes in the hydrogen-ion concentration of the medium was described by KOFFMAN (1924). DARBY (1929), working with *Stylonychia pustulata*, reported similar results. HOGUE (1915) concluded that metabolic wastes and O_2 deficiency both operate in inducing encystment in *Amoeba*. MAST and IBARA (1923) regarded metabolic wastes as playing a primary role in the encystment of *Didinium*. PENN (1935) also found that the excretion products of a number of organisms facilitated the encystment of *Pleurotricha*.

One finds in the literature on protozoan encystment several suggestions as to the role or roles of encystment in the life-history of the organism; and the factors which cause encystment, as discussed in the various reports, are more numerous. Perhaps one should not expect to find uniformity in the role of encystment and in the factors which induce it in the great diversity of forms which have been studied. The confusing thing, however, is the lack of agreement one finds concerning the factors which cause encystment in different studies made on the same organism.

At least one function of the thick-walled resting cysts in the life-history of *Woodruffia metabolica* is that of protection against certain adverse environmental conditions. Experiments to be reported later show that such cysts are able to retain their viability after being subjected to high evacuation, to extremes of desiccation, and to high and low temperatures. Our observations on the induction of encystment by lack of food have led to the questions — what other factors will induce encystment in this organism, and can the cause of encystment in this form be associated with adverse environmental conditions in general (having in mind that the cause or causes of encystment is synonymous with the factor or factors which induce it and not with the role or function it plays in the life-history of the organism). The experiments reported here were designed to answer, at least in part, these questions.

Materials and methods.

The *Woodruffia* used in all the experiments were derived from a single organism obtained from a stock culture grown in this laboratory for the past two years (cf. JOHNSON and LARSON, 1938). Members of the clone were supplied *paramecia* as food. Stock cul-

tures of *Paramecium multimicronucleata* were grown in infusions prepared by boiling $2^1/_2$ grams of dried timothy hay and 30 grains of wheat in a liter of tap water. This boiled preparation was poured into small wide-mouthed bottles and seeded with *paramecia* and *bacteria*. The clone of *Woodruffia* was subcultured daily by transferring a few to culture dishes containing many washed *paramecia* in balanced salt medium. When large numbers were needed for an experiment, several hundred *paramecia* were added to a one-day old *Woodruffia* culture and this was allowed to stand an additional day. In this way an adequate supply of experimental animals could be procured.

Balanced salt solution was prepared with triple glass distilled water and C. P. salts (Merck). The method for preparing the salt has been described elsewhere (Johnson, 1933). The salt solution was buffered at a p_H of 6.9—7.0. Unless stated otherwise, the balanced salt medium was used in all the experimental cultures; and the organisms, both the *Woodruffia* and the *paramecia*, were washed in this solution before their transfers to the experimental dishes. Elodea solution, the excystment medium, was prepared by boiling 3 grams of the dried plant in 500 cc. of balanced salt medium and then autoclaving. This amount was sufficient to last throughout the experiments.

Columbia watch glasses were used as culture dishes. Following each experiment the dishes were scoured thoroughly with a cellulose sponge, rinsed in distilled water, and dried with a clean towel. Petri dishes were used as moist chambers. Constant temperature rooms were used for experiments conducted at temperatures of 10° C, 15° C, and 20° C. Incubators were used for experiments conducted at all higher temperatures. Constant temperature rooms were maintained at $\pm 0{,}1^\circ$ C, and incubators at $\pm 0{,}5^\circ$ C.

When required, sterile salt solution was prepared by autoclaving at 15 lbs. pressure for 15 minutes. Petri dishes, pipettes and culture dishes were sterilized in a dry oven at a temperature of at least 163° C for $1^1/_2$ hours.

The various technical methods will be discussed in their respective sections in the body of the paper.

Woodruffia metabolica forms three distinct types of cysts. One type is the division cyst with a thin gelatinous wall easily ruptured. A second kind is the one we refer to as the permanent resting or protective cyst. The cyst wall of this type is thick and rigid, resistant to mechanical injury and to many adverse conditions of

the environment. These resting cysts do not excyst unless some plant extract is added. A third type of cyst is produced in thriving cultures with plenty of food present. Such cysts, when an excess of food is present, may be more numerous than the free-swimming individuals. The cyst wall resembles that of the division cysts, being thin and gelatinous. If such a cyst wall is ruptured, the organism swims out behaving the same as a free-swimming form. In some preliminary studies it has been found that each time a *Paramecium* has been engulfed, the *Woodruffia* usually forms a cyst of this type, the duration of which is about one-half hour, the approximate time required for digestion and assimilation of the food. Upon emergence from this type of cyst, the quest for more food is continued.

At the outset, it was obvious to us that suitable food was a most essential requirement for the maintenance of the free-swimming state. Without a single exception encystment is 100 per cent within a few hours when the food supply is depleted in a culture. The pre-encystment period may vary with the amount of unassimilated food in the body; consequently, so far as this factor is concerned, it seemed desirable that organisms in as nearly the same condition as possible be used.

In the life history of *Woodruffia* there appeared to be a stage eminently suited to our purpose, namely, the third type or temporary resting cyst referred to above. It was found that if approximately the same sized cysts of this type were removed from their source of food, their encystment time was always constant under a given set of conditions.

Numerous measurements have shown that the permanent resting cysts are quite constant in size, i. e., these cysts are far more constant in size than are the free-swimming forms. By a few preliminary experiments with isolation cultures it was found that *Woodruffia* measuring 300 μ or more in length usually divide twice to form four individuals, before the formation of resting cysts. Organisms measuring between 250—300 μ in length usually divide once before encysting. After some practice it was possible to pick out temporary resting cysts under a dissecting scope which would divide only once, and in all encystment experiments where the encystment time was involved, organisms of this size range were selected. The thin cyst walls of these cysts were ruptured during the first wash giving free-swimming organisms for the experiments.

By following the life-history of one organism in a hanging drop culture containing a limited number of *paramecia* (4—8) it was possible to determine the approximate number of *paramecia* required for a single division of a *Woodruffia*. It was found, after a number of trials, that usually two average-sized and adequately fed *paramecia* were sufficient to allow one division of a *Woodruffia*. Consequently, if a culture of about 1,000 *paramecia* is inoculated with one *Woodruffia*, the number of free-swimming *Woodruffia* when all the food is gone will be about five hundred, and the number of permanent resting cysts will total nearly one thousand. Undernourished *paramecia* have a striking effect on the reproduction and encystment of *Woodruffia* which will be discussed in a later section of the paper.

Effects of hydrogen-ion concentration.

Three sets of buffer mixtures were prepared as described by CLARK (1925). These buffer mixtures with the p_H range over which they were used are as follows: acid potassium phthalate, p_H 5.0—6.0; acid potassium phosphate, p_H 6.0—8.0; boric acid-potassium chloride, p_H 8.0—9.5. To a 50 cc. volume of balanced salt solution were added 1.2 cc. of M/10 buffer solution. The p_H was regulated with M/10 NaOH. The 50 cc. buffered balanced salt preparations were used as stock solutions. The increment in hydrogen-ion concentration was 0.5 of a p_H unit, except in the region of the acid death point where it was 0.2 of a p_H unit. La MOTTE's colorimetric standards were used in determining the p_H.

In the first experiment with different H-ion concentrations both the *Woodruffia* and the *paramecia* used were washed through two baths of the balanced salt solution. Following this, groups of three *Woodruffia* were washed in 1 cc. of the salt solution buffered at the p_H at which each group was to be tested, and the groups of three were then transferred with a minimum of the solution to culture dishes. To each dish were added 100 *paramecia* which had also been washed in salt solution buffered at the p_H to be tested, making the total volume of each culture about $^1/_4$ cc.

Throughout the range of p_H 5.5—9.5 the *Woodruffia* reproduced and encystment did not occur until the food was used up. This was repeated nine times with similar results. The fission rates were quite similar throughout this range. Table 1 gives the results. At p_H 5.0 both the *Woodruffia* and the *paramecia* died without division; and there was no cyst formation in the *Woodruffia*. Tests were

then made to see whether the H-ion concentration of the medium would affect the rate of encystment in the absence of food.

Table 1.
Fission rate at different H-ion concentrations.

pH	Average daily fission rates for 9 trials	pH	Average daily fission rates for 9 trials
5.0	0.*	7.5	2.58
5.5	2.92	8.0	2.58
6.0	3.04	8.5	2.24
6.5	2.33	9.0	2.42
7.0	2.5	9.5	2.16

* died.

Sufficient *Woodruffia* for one experiment were selected from the stock clone and transferred through two baths of balanced salt. Ten individuals, which constituted a single experimental culture, were then transferred with as little fluid as possible (1/100 cc.) through 1 cc. of the balanced salt buffered at the p_H at which this group was to be subjected. From here they were removed with a minimum of fluid to the experimental dish to which was added 1 cc. of the buffered solution. These cultures were kept in an incubator with a temperature of 26° C.

Under these conditions, without food, each *Woodruffia* divided once before the formation of permanent cysts. In all cases encystment was 100 per cent except in the immediate range of the acid death point. Table 2 shows that encystment is neither facilitated nor inhibited over a very wide p_H range — 5.6—9.5. At p_H 5.0, encystment never occurred. Some of the *Woodruffia* died almost immediately; others remained alive for as long as 24 hours, often fragmenting. If the fragments were transferred to a favorable medium, the nucleated portions formed normal cysts while the enucleated portions soon died. At p_H 5.2 the same general results were encountered with the exception that fewer organisms died immediately; it was, however, definitely toxic. Few, if any, deaths occurred at p_H 5.4, but when allowed to stand for 24 hours, by which time the H-ion concentration had decreased to p_H 5.6 or less, cysts appeared. In the same way some cysts were formed in the cultures started at p_H 5.2 after 48 hours. Encystment is definitely inhibited at p_H 5.2 and p_H 5.4. It generally required 24—48 hours for the H-ion concentration to change 0.2 to 0.4 p_H units and not until then did cyst formation occur. Beers (1927) has demonstrated that *Didinium*

possesses the identical acid tolerance that we have found in *Woodruffia*. At p_H 5.6 the encystment time was slightly longer on an average.

Table 2.
Rate of encystment at different H-ion concentrations

pH	Encystment time in hours					
	Buffer	Exp. I	Exp. II	Exp. III	Exp. IV	Average
5.0	phthalate	death	death	death	death	
5.2	"	*	*	*	*	
5.4	"	*	*	*	*	
5.6	"	9.0	9.5	9.25	9.5	9.3
5.8	"	7.5	8.5	8.0	8.0	8.0
6.0	"	8.25	8.5	8.25	7.5	8.12
6.0	phosphate	8.75	9.0	8.5	8.0	8.5
6.5	"	8.0	8.25	8.5	8.5	8.3
7.0	"	8.5	8.5	8.0	8.5	8.4
7.5	"	7.75	8.5	8.0	8.0	8.1
8.0	"	8.25	8.0	7.5	8.0	7.9
8.0	borate	8.75	8.75	8.0	7.5	8.2
8.5	"	8.25	8.5	8.0	7.5	8.1
9.0	"	8.5	8.25	7.5	8.25	8.1
9.5	"	8.5	8.0	7.75	8.25	8.1

* Encystment inhibited until pH falls to 5.6.

Effects of increased salt concentration of the medium.

Cienkowski (1855) improvised a moist chamber with which to observe protozoan cultures over long periods of time. As the small cultures evaporated he observed encystment and described it. When he added water to his dried preparations, excystment occurred. Other investigators have used this same method, evaporation, to induce encystment. Gradual evaporation was the method employed by Garnjobst (1937) for inducing encystment in *Stylonethes sterkii* and *Euplotes taylori*. Mélant (1922), by increasing the salt content or by adding sugar, caused encystment in *Euplotes harpa* and concluded that osmotic pressure was the activating agent in cyst formation. According to Schewiakoff (1928), *Euplotes harpa* may be induced to assume various cystic forms, depending on the rate of evaporation. If the process is rapid the organisms merely come to to rest without secreting a wall. By less rapid evaporation, the organisms rounded up but only a part of them formed a thin cyst membrane. By slow evaporation, occupying two to three days, the organisms always formed a rigid cyst wall.

To determine whether evaporation was effective in inducing encystment in *Woodruffia*, one organism was placed on a glass slide

in about $^1/_5$ cc. of balanced salt containing many *paramecia*. To prevent too rapid evaporation the slide was placed in a moist chamber at room temperature. After two days the volume of the culture had been reduced sufficiently to compress the *Woodruffia* and the *paramecia*, and complete drying occurred during the third day. By the end of this time the *Woodruffia* had multiplied to many, perhaps 75. Abundant food was still present. In several attempts not a single case of encystment was found unless the food had all been consumed before the end of the second day.

Probably one of the important factors which varies during evaporation is the salt concentration. A number of balanced salt solutions were prepared by diluting 6 per cent OSTERHOUT's solution with second glass distilled water. The concentrations were as follows: 0.012 %, 0.024 %, 0.06 %, 0.12 %, 0.24 %, 0.6 %, 1.2 %, and 1.44 %. A large number of *paramecia* were washed by centrifuging. Groups of approximately 100 of these were transferred with micropipettes through two baths of each of the different salt concentrations and then transferred with about $^1/_{20}$ cc. of the medium in the last wash to an experimental dish. *Woodruffia* were similarly washed and three of them were placed in each of the experimental dishes. In concentrations of 1.44 % and 1.2 % salt all the *paramecia* died within one hour. In the 0.6 % cultures a few *paramecia* died. Few *Woodruffia* survived 1.44 % salt and those that did encysted after about 24 hours. The *Woodruffia* were not killed in the 1.2 % salt. They did not utilize the dead *paramecia* as food but encysted without division to form three large viable resting cysts in all of the different trials. In all lower concentrations the *Woodruffia* fed and multiplied normally until the food was gone. In distilled water both *paramecia* and *Woodruffia* died.

Table 3.

Effects of salt concentration on encystment time.

Concentration of salt	Encystment time in hours				
	Exp. I	Exp. II	Exp. III	Exp. IV	Average
dist. water	death	death	death	death	
.012%	8.0	8.0	9.0	8.25	8.3
.024%	8.0	7.5	8.5	8.0	8.0
.06 %	7.5	7.5	8.0	7.5	7.6
.12 %	7.5	7.5	7.5	7.5	7.5
.24 %	8.0	8.5	8.5	7.5	8.1
.60 %	8.0	8.5	9.0	8.5	8.5
1.2 %	18.0	16.0	15.0	20.0	17.2
1.44 %	death	death	death	death	

Table 3 shows that all concentrations of salts between 0.012 and 0.6 % inclusive do not influence the rate of encystment of *Woodruffia* when food is absent. In each case ten *Woodruffia* were placed in each experimental dish and kept at 26° C. The average encystment time of the controls (0.012%) was slightly longer than 8 hours. The average encystment time in the 1.2 % cultures was 17.2 hours; thus instead of facilitating encystment in this form, this greater concentration of salts is inhibitory to a marked degree. Strangely, however, the *Woodruffia* in this concentration of salts seldom divided before encysting and the cysts averaged 15 μ larger in diameter than those in lower concentrations. An occasional death and an occasional division occurred. In 1.44 % salt, almost all of the *Woodruffia* died without encysting.

Effects of temperature, crowding and the excystment medium.

The effects of temperature, crowding and the excystment medium (Elodea solution) on the rate of encystment are shown in Table 4. Three experiments for each condition were conducted. The results, in hours required for encystment, of each experiment and the aver-

Table 4.

Effects of temperature, crowding and the excystment medium.

Temp.	Elodea solution				Salt solution			
	1/50 cc.		1 cc.		1/50 cc.		1 cc.	
	10 org.	1 org.	10 org.	1 org.	10 org.	1 org.	10 org.	1 org.
34°	8.0	9.0	8.5	11.0	4.5	6.0	6.5	9.0
	7.5	10.0	11.0	9.0	5.0	7.5	6.5	8.5
	8.5	10.0	12.0	15.0	5.0	7.0	6.5	8.5
Av.	8.0	9.6	10.5	11.6	4.8	6.8	6.5	8.7
26°	8.0	11.0	12.0	14.0	6.5	8.2	8.0	14.0
	8.0	11.0	12.0	14.0	7.5	8.0	9.0	11.5
	10.0	13.0	13.0	16.0	7.5	9.0	8.5	12.0
Av.	8.7	11.7	12.3	14.7	7.2	8.4	8.5	12.5
20°	12.0	16.0	17.0	26.0	9.0	15.0	11.5	26.0
	12.0	17.0	15.0	35.0	9.5	14.0	14.0	18.0
	12.0	18.0	17.0	28.0	9.0	13.0	12.5	17.0
Av.	12.0	17	16.3	29.7	9.2	14	12.7	20.3
15°	19.0	28.0	30.0	30.0	16.0	28.0	24.0	29.0
	28.0	30.0	27.0	32.0	20.0	26.0	25.0	30.0
	24.0	28.0	28.0	34.0	19.0	26.0	28.0	32.0
Av.	23.7	28.7	28.3	32	18.3	26.7	25.7	30.3

ages are given in the table. The *Woodruffia* were washed and placed in the media without food. The results show that at the temperatures used the process of encystment occurred most rapidly at the highest temperature (34° C) and that it required the longest time at the lowest temperature (15° C). Also, a longer time is required for encystment in the Elodea medium than in the corresponding cultures in salt solution. Fig. 1 shows graphically the results of the two kinds of cultures when 10 organisms in 1 cc. of medium were used. An examination of Table 4 will show that similar results were obtained in the other phases of the experiment.

It is not clear just why the organic excystment medium retards the process of encystment. There was considerable growth of bacteria in these cultures, but whether this affected the process of encystment is not obvious. In numerous attempts over the past two years to grow this organism on different kinds of bacteria suspended in salt solution, no indication has been obtained that they utilize bacteria as food. In fact, encystment occurs just as quickly in the various suspensions of bacteria tried as in the salt medium free of bacteria. It may be that the excysting substance in the solution somehow hinders encystment. Also, it may be that the *Woodruffia* utilize something in the solution as nourishment and that this prolongs the free-swimming state. Reference will be made to this again in the section on the use of sterile organisms in sterile Elodea solution.

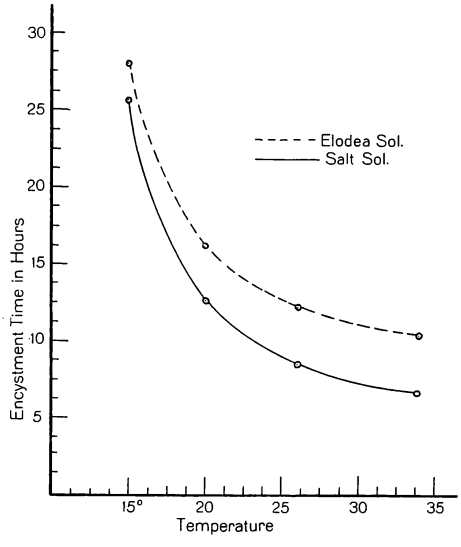

Fig. 1. Effects of temperature on encystment time in Elodea and salt solutions using 10-animal cultures in 1 cc. encystment medium.

That the number of organisms in a given volume of medium or the amount of medium per individual in a culture, i. e., crowding, affects the rate of encystment is also shown in Table 4. Fig. 2 shows this relationship graphically. A complete analysis of this effect is not possible at the present time. However, we do have

some evidence which indicates that part of the crowding effect, at least, is due to a touch or contact stimulus.

Washed *Woodruffia* were placed in small drops of salt solution ($^1/_{50}$ cc.) in groups of 10, 20, and 30, and also singly. In addition to these, single organisms were placed in similar drops which contained clumps of finely cut cotton fibers. Tests for encystment time were made at both 20° and 26° C. Table 5 shows the results of this experiment. The time given in each case is the average of four trials. There was no difference in encystment at any one temperature in the groups of 10, 20 and 30 organisms. The organisms isolated singly without cotton fibers in the culture required the longest time for encystment, while those isolated singly with a clump of cotton fibers in the medium encysted on the average almost as rapidly as the grouped organisms and these *Woodruffia* always encysted in contact with a cotton fiber. When numerous *Woodruffia* are present in a culture resting cysts are always formed in dense clusters. This behavior with the cotton fibers seems to indicate that contact with something in the environment facilitates encystment. The fact that we have been unable to induce encystment in *Woodruffia* by crowding when food is present in abundance in the balanced salt culture medium leads to the conclusion that crowding does not induce encystment under the culture conditions we have used; but with these same conditions, and in the absence of food, a certain amount of crowding or contact with something in the environment shortens the encystment time.

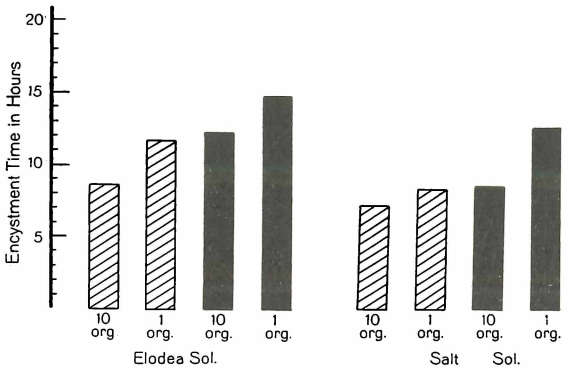

Fig. 2. Effects of crowding in Elodea and salt solutions on encystment time at 26° C. 1. Cross-hatched — $^1/_{50}$ cc. volumes. 2. Solid bars — 1 cc. volumes.

Attempts were made to induce encystment with extremes of temperature, both high and low. Ten washed *Woodruffia* were placed in 1 cc. salt cultures containing approximately 200 washed *paramecia*. Temperatures of 39° and 40° C proved to be lethal to

Table 5.

Influence of cotton fibers on encystment.

No. of organisms	Hours for encystment at 26° C.	Hours for encystment at 20° C.
30	6.75	9.5
20	6.75	9.5
10	6.75	9.5
1	9.6	14.2
1*	7.0	9.75

* With cotton fibers.

both the *Woodruffia* and the food organisms. At 40° C the *paramecia* were all killed within an hour while some of the *Woodruffia* lived a little longer. At 39° C the *paramecia* were all killed within two hours while some of the *Woodruffia* lived for about 24 hours. No cysts were formed. At 38° C some of the *paramecia* lived as long as 5 hours, and, although there was death of some *Woodruffia* at this temperature, some survived and a few viable cysts were formed after three days. A temperature of 37½° C did not kill the *paramecia*, and the *Woodruffia* in these cultures consumed all of the food before forming resting cysts. All of these cysts were viable. This indicates that high temperature does not induce encystment. Even though the *Woodruffia* were able to survive somewhat longer than the food organisms at these upper critical temperatures, the formation of viable cysts was not possible above 38° C. At 37½° C, where the process was normal in that the *Woodruffia* consumed the food before encysting, the encystment time was somewhat longer than that found in more optimum temperatures.

By use of a cold chamber[1]) it was possible to subject *Woodruffia* to a temperature of less than 1° C and keep them at this temperature for an hour without death. It was necessary to reduce the temperature gradually over 1½—2 hours, as quite rapid lowering of the temperature caused the death of most of the organisms. After an hour at this temperature the organisms were still free-swimming although their movements were quite sluggish. The chamber containing the *Woodruffia* was then placed in an electric refrigerator at 4° C. Here some of the organisms lived as long as five days.

[1]) We are indebted to Dr. C. V. Taylor for the use of this chamber which he devised for use on the stage of a microscope. The temperature was regulated by adjusting the flow of iced brine through the hollow walls of the chamber. Temperature readings were taken with a thermometer inserted into the chamber with the bulb in contact with the medium in the chamber containing the organisms.

At the end of that time no cysts had been formed. Some of the *Woodruffia* were removed from the cold chamber at the end of the first and second days and left at room temperature. They formed normal viable cysts in each case. This experiment was repeated three times with similar results. Death of both the *Woodruffia* and the *paramecia* did not occur with fragmentation and cytolysis. Instead the organisms rounded up and remained whole for some time.

In another attempt to see whether low temperature would induce encystment, the temperature was lowered on three successive days. Three dishes containing 1 cc. of washed *paramecia* (approximately 300) and 10 *Woodruffia* were placed in a constant temperature room at 15° C for 24 hours. At the end of this time two of the dishes were removed to a second cold room at 10° C for another 24 hours. Then one of these dishes was placed in a refrigerator at 4° C. The course of these cultures was then followed. At 4° C all of the *Woodruffia* and *paramecia* were dead at the end of five days; no cysts were formed. At 10° C the organisms were quite sluggish and growth was slow. At the end of 9 days there were 56 *Woodruffia* and no resting cysts. On the fourteenth day there were 91 free-swimming *Woodruffia* and 19 resting cysts. By this time there were few *paramecia* left. On the sixteenth day all the *paramecia* were gone and only resting cysts were present. At 15° C no resting cysts were formed until the end of the fifth day when the food was quite scarce. By the end of the sixth day all had encysted.

This shows that a temperature of 4° C prevents cyst formation and gradually causes death. Encystment occurs in the normal manner at 10° and 15° C and the cysts are viable. At 10° C a few cysts were formed before all of the *paramecia* had been consumed, something which does not occur at higher temperatures. However, the *paramecia* were quite sparse in the culture at that time and the movements of the *Woodruffia* were so sluggish that it appears likely that those which encysted were unable to find food.

At 15° C about twice the number of cysts were formed as in similar 10° cultures. However, most of the cysts formed at 10° C were over 100 μ in diameter. Those formed at 15° C were normal in size (50—75 μ in diameter). Apparently at 10° C most of the free-swimming forms fail to undergo the normal division before cyst formation. This would account for the large size of the cysts.

Temperatures, both high and low, which are lethal to free-swimming *Woodruffia* prevent the formation of resting cysts in the tests we have made.

Effects of starved *paramecia* as food.

Beers (1928) reports that when *Didinium* is fed starved *paramecia*, they gradually lose their ability to form resting cysts. This was tested on *Woodruffia*. Thousands of *paramecia*, after being washed in balanced salt solution by centrifuging, were placed in a flask of the salt solution without food for 14 days. The *paramecia* at the end of this time were definitely in a state of starvation. They were slender and transparent; their average size was 140—160 μ, while the well fed *paramecia* used in the control experiments were full of food vacuoles and averaged 200—250 μ in length. Six paired experiments were started on successive days. Approximately 1 cc. of *paramecia* in balanced salt solution was placed in each culture dish and each dish was inoculated with three *Woodruffia* of the same size and age. The number of *paramecia* in the culture dishes varied from approximately 250 to 1,000 on the different days, but the variation in numbers of *paramecia* in the control dish and the experimental dish was slight on any given day. The cultures were kept at 26° C.

Table 6.

Effects of starved *paramecia* as food.

Series	Control			Experimental		
	No. *Woodruffia* first 24 hours	Cysts at end	Duration in days	No. *Woodruffia* first 24 hours	Cysts at end	Duration in days
1	19	1100	3	18	8	7
2	38	550	5	19	7	8
3	5 cysts *	350	5	17	3	7
4	27	500	4	18	0	8
5	23	225	4	15	1	7
6	19	300	4	13	2	10

* No free-swimming *Woodruffia* for unknown reasons. Cysts replaced with 3 free-swimming organisms.

Table 6 shows the results of this experiment. Accurate counts were made on the free-swimming forms only after the first 24 hours. The number of cysts listed in the control cultures indicates the number of organisms formed in each control culture. Cysts did not form in the control cultures until the food had been depleted. The duration in days of the control cultures gives the time required to use up the food and start encystment. The numbers of *Woodruffia* in the experimental dishes at the end of the first day do not represent the maxima attained in these dishes; by the end of the

second day most of these dishes contained from 50 to 60 *Woodruffia*. Thereafter, on each succeeding day, there were fewer *Woodruffia* in each culture. All of the *Woodruffia* were either dead or had formed cysts one or two days before the cultures were terminated. The duration in days of the experimental cultures indicates the time when all of the *paramecia* had died. In all of these cultures the *Woodruffia* had either died or formed cysts before the *paramecia* had all disappeared. The *paramecia* apparently died of starvation, having been without food for three weeks or more in some cases.

The number of cysts formed in the experimental cultures was small in each case and these results indicate that a diet of starved *paramecia* greatly impairs the ability of *Woodruffia* to form resting cysts. This is similar to the finding of Beers on *Didinium*. The small number of cysts so formed were tested for excystment, and 75 % of them proved to be viable cysts.

Encystment in sterile and bacterized Elodea solution.

In a previous section of this paper it was shown that encystment in Elodea solution was somewhat prolonged over that in the salt controls. These experiments were designed to find out if bacteria would remove the substance or substances from the Elodea medium which delayed encystment. One of three test tubes containing sterile Elodea solution was inoculated with a mixed culture of bacteria from a hay infusion, another with *Pseudomonas fluorescens* and the third was used as a control. The three solutions were incubated at a temperature of 30° C for two weeks. At the end of this time the bacterized solutions were filtered through Chamberland-Pasteur porcelain filters and autoclaved.

The experimental animals were prepared by washing several times in sterile balanced salt solution. The pipettes, culture dishes and petri dishes used were all sterile. From the last wash the organisms were transferred to the encystment solution. The organisms used in the salt controls were similarly washed. However, the encystment time was identical in sterile and non-sterile salt as numerous trials showed. There was no apparent effect even when as many as 12 loops of bacteria per 5 cc. were added to the salt solution.

Table 7 shows that in the 10-animal cultures the encystment time is about the same for the salt control as for both bacterized solutions. Encystment in the unbacterized Elodea solution was significantly longer, requiring six hours longer on an average. In

Table 7.
Encystment time in bacterized and sterile Elodea medium at 26° C.

Medium	Hours for encystment with 10 organisms in 1 cc.	Hours for encystment with 1 organism in 1 cc.
Elodea with mixed bacteria	9.0	15.0
	9.5	14.0
	9.0	12.0
	Av. 9.2	Av. 13.7
Elodea with *Pseudomonas fluorescens*	8.5	13.0
	9.0	15.0
	9.5	16.0
	Av. 9.0	Av. 14.6
Sterile Elodea*	15.0	Usually dead after three days
	13.0	
	17.0	
	Av. 15.0	
Salt solution	8.0	14.0
	9.0	11.5
	8.5	12.0
	Av. 8.5	Av. 12.5

* In all 10-animal cultures in Elodea solution some were swimming after 24 hours. Encystment time is for 50% encystment.

the single-animal cultures the bacterized Elodea solutions delayed encystment two hours longer than in the salt control.

Encystment occurred in the single-animal unbacterized Elodea cultures in only one instance out of four cultures which remained clear and unclouded by bacterized growth for a period of three days. In several cases the medium became cloudy with bacteria after a day, and in these the *Woodruffia* always encysted. In the other three cultures the *Woodruffia* remained as free-swimming organisms for three days and then died. The fact that death occurred after 3 days without encystment indicates that something in the solution inhibited encystment. This might be an effect of the excysting substance in the Elodea but we have no evidence on that. Numerous investigators have been able to grow certain protozoans in organic solutions free of other living organisms. Taylor and Strickland (1938) found that *Colpoda duodenaria*, when made sterile and placed in sterile yeast extract (their excystment medium), could apparently utilize something in the yeast extract as food, grow, and in some cases even divide before forming resting cysts. In several attempts to grow sterile *Woodruffia* in sterile yeast autolysate, so far, we have had no success. However, single organisms placed in small

volumes of the autolysate usually remained in the free-swimming state for about one week. This shows that they receive some nourishment from the autolysate. At present we do not know whether such organisms encyst or die. Thus far no cysts have been found.

When these results are compared with those in the preceding section on the effects of starved *paramecia* on the encystment of *Woodruffia* certain similarities will be noticed. The *Woodruffia* feeding on starved *paramecia* multiplied some, but most of them died without encysting, apparently because this diet is deficient. In these cultures the *Woodruffia* maintained the free-swimming state for three days — something which, under other circumstances, never happens in the absence of the food organisms — and then died. If we can assume that the *Woodruffia* obtain some nourishment from the Elodea medium which keeps them in the free-swimming state for a few days, but that this food is quite inadequate and thus leaves the *Woodruffia* incapable of forming cysts, then the situation in the sterile Elodea cultures containing one organism is comparable in some respects to that where the *Woodruffia* were fed on starved *paramecia*.

Whatever the substance is which is effective in inhibiting encystment in the sterile Elodea medium, it is not strong enough to overcome the contact stimulus exhibited in the 10-animal cultures in the same medium. The results show that bacteria do change or remove the substance from the Elodea medium which delays or inhibits encystment in *Woodruffia*.

Metabolites and encystment.

Metabolites seem to exert little influence on the encystment of *Woodruffia* as shown in Table 8. Metabolites of *Woodruffia*, *Paramecium* and the bacterium *Pseudomonas fluorescens*, were tested. The metabolite solutions were prepared in the following manner. Metabolites of *Woodruffia* were accumulated by washing approximately 200 organisms three times in balanced salt by transferring with a micropipette. From the last wash they were removed to a watch glass containing 1 cc. of balanced medium and allowed to remain until encystment commenced. These were removed and a fresh supply, prepared in the same way, added. This procedure was performed twice each day for five consecutive days. Metabolites of *Paramecium* were accumulated by seeding a 500 cc. flask containing balanced medium with a few *paramecia*. Five loops of *P. fluorescens*

taken from an agar plate were added to this culture daily. The population of *Paramecium* rose rapidly to approximately 250 individuals per cc. and it was maintained at this level for two months. The *paramecia* and bacteria were removed by passing the liquid through a porcelain filter, and this liquid was used. Two other flasks were prepared in the same manner. When the populations had reached their peaks, the *paramecia* from each flask were concentrated by centrifuging, washed in fresh salt solution, and finally concentrated in 10 cc. of the salt medium where they were left for one week at 26° C. This preparation was made because the one from the clone culture in the first flask contained not only metabolites from the *paramecia* but also from the bacteria. The *paramecia* were removed from this liquid by passage through filter paper. Metabolites of *Pseudomonas* were collected by making a dense suspension of the bacteria in 20 cc. of sterile salt medium. Twice each week more bacteria were added over the course of a month. The bacteria were finally removed by passage through a porcelain filter.

Table 8.

Effects of metabolites on encystment time.

Encystment medium	Encystment time in hours	Encystment medium	Encystment time in hours
Salt control	8 5	Centrifuged *Paramecium* metabolites	7.5
	7.5		8.5
	8.0		9.5
	9.0		8.0
	8.5		Av. 8.4
	Av. 8.2		
Paramecium clone metabolites	10.5	*Pseudomonas fluorescens* metabolites	9.5
	9.75		9.5
	9.5		9.0
	9.0		9.5
	8.75		Av. 9.4
	10.0		
	10.5	Washed *Woodruffia* metabolites	7.5
	Av. 9.7		7.5
			6.5
			6.5
			Av. 7.0

The experimental animals were washed in salt solution and 10 animals were transferred to 1 cc. of each of the metabolite preparations and allowed to encyst at 26° C. The results given in Table 8 show that these waste products, so collected, have little effect in either facilitating or inhibiting encystment in the absence of food. *Woodruffia* metabolites seem to facilitate encystment slightly and *Pseudo-*

monas metabolites to inhibit it slightly. *Paramecium* metabolites have no effect.

With each of the metabolite preparations used, control dishes containing numerous *paramecia* in the metabolite solutions were run. Here as in the other experiments where the salt solution was used, no encystment occurred until the food had been depleted.

Use of old *Paramecium* infusions.

In maintaining stock cultures of *Woodruffia* some of the cultures were carried along by using *paramecia* taken directly from hay infusion cultures. In these cultures the accompanying infusion liquid constituted the medium for the *Woodruffia*. In such stock cultures occasionally a few resting cysts were formed before the food had been used up. This seemed to be associated with the use of old infusions. Such infusions can be maintained several months by adding fresh medium at intervals to make up for losses due to evaporation and removals. Accordingly, a series of tests was made to see whether this sporadic appearance of resting cysts could be associated with the age of the infusions, and also to ascertain, if possible, the cause.

Nine cultures with each of the following kinds of medium were tested, three of each on three successive times: 1-week infusion, 2-weeks infusion, 1-month infusion, 2-months infusion, 4-months infusion, filtered 2-months infusion, filtered 4-months infusion, and the salt solution as a control. The filtered medium was prepared by passage through a porcelain filter. These cultures were kept at a temperature of 26° C.

No encystment occurred in any of the cultures except some of those in which unfiltered 2- and 4-months old infusion was used. Out of the nine cultures with the 2-months old infusion, cysts formed early in three of them and the percentage of cysts so formed was 0.6 % of the total formed. In the 4-months old infusion cultures a few cysts formed early in five of the nine cultures. Here the percentage of the cysts formed in the presence of food was 3.0 % of the total formed in all of the dishes. Further tests were made using *paramecia* taken from the 2- and 4-months old infusions which were washed in salt solution and then placed in fresh salt solution for the *Woodruffia* cultures. In several such tests no cysts were formed before the *paramecia* were used up. This shows that the *paramecia* from such cultures are satisfactory as food. The facts that no encystment occurred prior to the depletion of the food when

filtered infusion liquid was used, and that when encystment did occur in those old infusions it did not occur in all of them, might indicate that the ingestion of particles of debris or some of the zooglea in such liquid was the cause of such encystment as occurred. It is not altogether improbable that some of this debris or zooglea might occasionally occlude the mouth of one of the *Woodruffia*. If this happens, the subsequent lack of nourishment would be the cause of encystment. A further study needs to be made of this. Only one test on the viability of these cysts, so formed, was made, and here the percentage of excystment was only 25 %.

Successive encystments without food.

When a 0.6 % solution of dried Elodea is placed on 3-day old resting cysts, they normally excyst 100 %. If no food is added for these excysted forms to feed on, they will form resting cysts again after several hours of swimming around in search of food. When fresh excystment medium is added to these second — formed cysts, they will excyst but as smaller organisms. It has been possible to carry a small number of *Woodruffia* through seven successive encystments and excystments without feeding. The size of both the free-swimming forms and the cysts decreases with each encystment and excystment. In the organisms followed, the average length of free-swimming forms at the start was 325 μ and only 50 μ after the seventh excystment. Such small organisms cannot ingest a *Paramecium* and at present we have found no way to feed them.

The normal time required for excystment at 26° C is 9 hours. The excystment time decreased with each successive excystment so that the seventh excystment required less than one hour. Accompanying this great decrease in size are certain structural changes which are being investigated at the present time.

Discussion.

The results obtained in these experiments with the use of a balanced salt culture medium and with wide variations in the various environmental factors which have been considered as causes of permanent cyst formation in various protozoa indicate that lack of food is the primary cause of the formation of permanent resting cysts in *Woodruffia metabolica*, when so cultured.

Over a wide range of hydrogen-ion concentrations this organism formed normal viable resting cysts, but only in the absence of food. In the absence of food the rate of encystment was not affected over

this same range except at the concentrations near the acid death point where the time was somewhat longer. There is much evidence presented in the literature that hydrogen-ion concentration does not affect encystment in a wide variety of protozoa. v. BRAND (1923) reports little effect of hydrogen-ion concentration on encystment in *Vorticella*. Similarly, KATER and BURROUGHS (1926) on *Polytomella*, BEERS (1927) on *Didinium*, WEYER (1930) on *Gastrostyla*, and PENN (1935) on *Pleurotricha* conclude that encystment is not induced by unfavorable hydrogen-ion concentrations. TAYLOR and STRICKLAND (1938), culturing *Colpoda duodenaria* in an unbuffered balanced salt medium, found that encystment occurred just as readily at hydrogen-ion concentrations approaching neutrality as those at 6.0 and 8.2, but only when the food supply was depleted. The conclusions of KOFFMAN (1924) on *Colpoda* and of DARBY (1929) on *Stylonychia* are to the effect that marked changes in the hydrogen-ion concentration in their cultures induced encystment. It must be borne in mind, however, that neither of these workers presented evidence of having the food in their cultures controlled.

It was not possible to induce permanent cyst formation in this organism either by slow evaporation of the medium or by increased salt content of the medium. Reference has been made (see above) to protozoa which have been induced to encyst by evaporation of the surrounding medium. It may be significant that all of these forms are either brackish or salt water organisms. To our knowledge, no fresh water species has been induced to encyst in such a way under controlled conditions. CIENKOWSKI's (1855) preparations contained fresh water protozoa and he implies that encystment was due to evaporation; however, his cultures were uncontrolled as to food and various other factors so that it is difficult to say what the activating agents were. GARNJOBST (1937) has suggested that increased salt concentration may be a contributing factor but states that she has made no study of the problem. MÉLANT (1922) has demonstrated that the marine ciliate, *Euplotes harpa*, can be induced to encyst by increasing the salt content. If such is the case, it may prove interesting to demonstrate the effect of this factor on various marine and brackish water protozoa under strictly controlled conditions.

Variations in temperature which were not lethal to the free-swimming organisms did not induce encystment when the *Woodruffia* were well supplied with food. Viable resting cysts were formed throughout a temperature range of 10° C to 37½° C. Temperatures above 38° C were always lethal. A temperature of 4° C killed *Wood-*

ruffia within five days. The actual lower limit where encystment can occur was not determined. The cysts formed at 10° C were considerably larger than cysts formed at higher temperatures. A similar situation existed in the cysts formed in the cultures with a 1.2% salt content. There is evidence that in each of these cases the organisms did not divide just prior to encystment as they usually do.

With abundant food present in the cultures it was not possible to induce encystment by crowding. However, in the absence of food a certain amount of crowding seems to speed up the encystment process. The fact that a single organism will encyst quicker when in contact with cotton fibers in the medium indicates that this crowding effect is probably due to a contact or touch stimulus.

Metabolites of *Woodruffia*, *Paramecium* and *P. fluorescens* collected in the salt medium did not induce encystment when food was present. In the absence of food the difference in rate of encystment was not very great in the different preparations. There is a slight indication that *Woodruffia* metabolites speed up the process, and that *Pseudomonas* metabolites slow it down; however, the differences in time are slight and as the times recorded were for 50% encystment in each case, we are inclined to feel that these differences are not very important.

The finding here that *Woodruffia* gradually lose their ability to form resting cysts and eventually die when fed on starved *paramecia*, is in essential agreement with the results of Beers (1928) on *Didinium*. It seems proper to attribute this to a qualitatively deficient diet. This is another instance where an adverse environmental condition does not induce encystment.

By the use of a non-nutritive inorganic salt medium it is possible to vary various environmental factors in a protozoan culture and analyze with some certainty the results. In so doing in this work it has been found that lack of food in a wide range of various culture conditions is the only important factor studied in the induction of encystment. O_2 deficiency has been suggested (Adolph 1929) as one possible cause of encystment. The O_2 content of the culture medium has not been varied in this work (all of the cultures were small in volume with a large surface exposed to the air) and thus this factor is not ruled out as a possible cause of encystment under other conditions. However, it can be said that lack of O_2 is not a necessary causal factor in the encystment of *Woodruffia*.

The analysis of encystment in the two kinds of organic medium used cannot be made with such certainty. The fact that there is

something in Elodea excystment solution which slows up the encystment process, something which can be altered by bacterial action, has already been discussed. This might be due to some effect of the excysting substance, although it seems more probable that the Elodea medium furnishes some nourishment for the *Woodruffia* and in so doing prolongs the free-swimming state. It is suggested that here, as in the experiments where starved *paramecia* were used as food organisms, a deficient diet makes the organisms incapable of cyst formation.

When unfiltered *Paramecium* infusion, 2- and 4-months old, was used as the encystment medium, a small percentage of resting cysts was found in some of the cultures. This did not happen when other infusions, not so old, were used. This may be due to the chance ingestion of particles of debris or zooglea present in such infusions; at least, no other explanation for such cyst formation seems a possibility at present. This is the only condition investigated in which resting cysts were formed in the presence of food. The percentage of cysts so formed was quite small, but it is recognized that the factor operating in this case may also operate in nature. The great majority of the cysts formed in these old infusions were formed after the *paramecia* had all been eaten. So here, as with the balanced salt cultures, the primary factor in cyst formation is the absence of food.

Although resting or protective cysts afford protection against many adverse environmental conditions, these experiments indicate that the cause of such cyst formation in *Woodruffia metabolica* cannot be attributed to adverse environmental conditions in general.

Summary.

1. When balanced salt solution was used as the culture medium for *Woodruffia metabolica*, resting or protective cysts formed only in the absence of food throughout a wide range of experimental conditions.

2. Viable resting cysts formed throughout a range of hydrogen-ion concentrations from p_H 5.6—9.5, but only in the absence of food.

3. Temperatures from 10° C to 37½° C allowed the formation of normal cysts. Temperatures of 4° C and 38½° C were lethal and no cysts were formed.

4. Viable cysts were formed in cultures varying in salt content from .012% to 1.2%.

5. Crowding and the use of metabolites collected in the salt medium did not induce encystment; however, there is evidence that a certain amount of crowding speeds up the encystment time in the absence of food.

6. The use of starved *paramecia* as food causes a gradual loss of the ability of *Woodruffia* to form resting cysts and ultimately leads to death.

7. A longer time is required for encystment in Elodea solution. A single sterile organism in sterile Elodea solution usually does not encyst but dies after three days.

8. Something present in 2- and 4-months old hay infusion sometimes causes the formation of a small percentage of resting cysts in the presence of food. This is the only instance studied where such cysts formed in the presence of food.

9. *Woodruffia metabolica* can encyst and excyst at least seven successive times without feeding. This results in a great reduction in size.

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