Reactions of *Colpoda duodenaria*[1] to environmental factors.

I. Some factors influencing growth and encystment.

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With 1 figure in the text.

The factors which control the encystment of protozoa have been studied by many investigators, but the conclusions reached have been often different and sometimes contradictory. The literature is so voluminous, so many kinds of protozoa have been the subject of study, and experimental conditions and technique have been so varied, that no attempt to review the literature will be made here. Reference may be made to the extensive bibliography by Penn (1934).

Among the factors acclaimed as inducers of encystment are the following: (1) food: deficient, sufficient, in excess; (2) excretion products: protozoan, bacterial; (3) desiccation of the medium: salt concentration; (4) lack of oxygen; (5) temperature: high, low, variation; (6) age of culture; (7) physiological periodicity; (8) hydrogen ion concentration; (9) crowding.

This paper deals primarily with the food factor, which in the case of *Colpoda* is of the greatest importance. Each of the other factors noted above has also been taken into account as possibly affecting the encystment of the organism.

[1]) It appears, Taylor and Furgason, Arch. Protistenkde (in press) that the ciliate used in the following studies: Taylor and Strickland, Arch. Protistenkde **86**, 1935 and Taylor and Strickland, Physiologic. Zool. **9**, No. 1, 1936, and therein called *Colpoda cucullus* should have been designated *Colpoda duodenaria*.

Material and methods.

Colpoda duodenaria grows very readily in an inorganic balanced salt medium with no organic constituent other than the suspension of bacteria which is added as required for food. The use of an inorganic medium excludes the many possibilities of error due to the growth of an unknown flora in an organic medium.

Colpoda duodenaria forms division cysts from which emerge 2, 4, or 8 daughters[1]), according to the size of the precystic animal. It also forms resting or protective cysts under certain incompletely determined conditions, and it is primarily the factors inducing encystment which are studied in this paper.

The protozoa were cultured in 500 cc. of the inorganic medium in an aeration flask at a constant temperature of 20° C. The medium was therefore continuously saturated with oxygen, the food held evenly in suspension, and the distribution of the free-swimming organisms uniform.

At the beginning of the experiment, which was run in duplicate, the 500 cc. of medium were seeded with 25 thousand *Colpoda*, i. e., 50 per cc.

The food, a pure culture of a selected *Pseudomonas* sp. (?) was added as required with a wire loop. There was, of course, some difference between loops of food, but not enough to show serious discrepancies in the results.

Figure 1 shows the growth and encystment curves obtained during the first two months, with a section obtained during the 15th and 16th weeks; the experiment is now at the end of its 4th month with no fundamental difference in results. The ordinates, on a logarithmic scale of several cycles, represent the number of free-swimming *Colpoda* per cc.; the abscissae, the time in hours from the seeding of the medium. Each observation (count) is represented by a small clear circle, each lopp of food by a large black circle. The rising curves indicate the increase by division in the number of free-swimming ciliates, the falling curves, a decrease due to the formation of resting cysts, with which the glass is now thickly encrusted. Once formed, these resting cysts do not excyst unless some special excystment medium, such as yeast extract, is added, so that each rising curve shows the increase by division of the remaining free-swimming organisms.

These rising curves do not show all the small fluctuations of the number of animals per cc. due to periodic and, to some extent,

[1]) Herein referred to as digenic, quadrigenic and octogenic cysts, respectively.

continuous encystment for division. The curves vary among themselves because the amount of food and the intervals between feeding were varied for experimental reasons. The rising and falling curves are not joined at the peak because the actual maxima were not ascertained by observation.

During the course of the experiment nothing was added to the medium except food, and nothing was removed except the few drops necessary to make the counts of the population, and such volatile

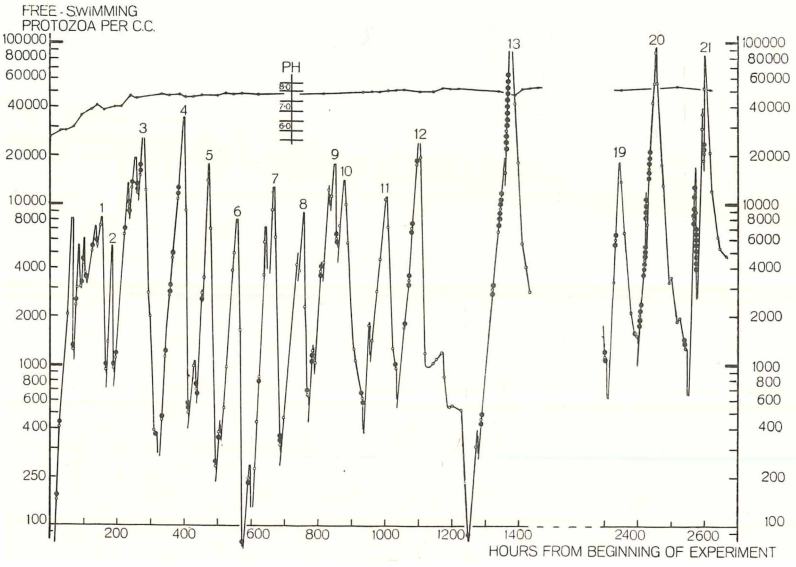

Fig. 1.
Increase and encystment of *Colpoda duodenaria* in relation to environmental factors.

metabolites, if any, as were removed with the aeration current. The air used for aeration of the medium was saturated with water vapor so that no evaporation of the medium occurred. No buffer was used, and the p_H of the medium was determined daily with a glass electrode by the potentiometric method.

Experimental.

Experiment I.

The first peak shows that when 10 loops of food were given at intervals over a period of 5 days the number of free-swimming *Colpoda* fluctuated, but with a rising tendency. Already large numbers of protective cysts were appearing on the glass.

When feeding was stopped at 140 hours, the surplus food was sufficient to send the population up to about 14,000 per cc. (Peak 1). It then, due to encystment, dropped in a few hours almost to 1,000 and would have gone lower if a loop of food had not been added. The concentration rose then to about 6,000 per cc. (Peak 2), followed by encystment.

At 1,000 per cc., a loop of food was added and at intervals on the rising curve 9 more were given. The maximum concentration of free-swimming organisms at Peak 3 was about 35,000 per cc. The near exhaustion of the food then induced precipitate encystment.

Eight more loops were added at intervals during the next period of increase and the maximum obtained at Peak 4 was about 40,000 per cc. With 5 loops the maximum was about 24,000 at Peak 5. With 2 loops the maximum was about 10,000 at Peak 6; and approximately the same ratio of food to maximal concentration is maintained throughout and is still being maintained after 16 weeks. Each loop of food produces about 5,000 *Colpoda* per cc., or 2,500,000 in the 500 cc. of medium. Peak 13, with a maximum of about 115,000 ciliates per cc., was produced with 23 loops of food.

In the figure are also shown three growth and encystment curves during the 15th and 16th weeks. They are of the same type as the preceding curves and show that the number of *Colpoda* produced per loop of food is still constant at about 5,000/cc. per loop. The intervening curves are omitted to save space; they do not show noteworthy differences from the others.

Experiment II.

In order to ascertain whether there is a limit to the concentration of *Colpoda* that can be obtained without inducing encystment, five drops of a culture were placed in a watch glass and supplied continuously with food. When finally the count was made, there were six million ciliates per cc. There was not one resting cyst; on the contrary, there was a large number of division cysts, showing that the maximum had not been reached.

Experiment III.

This experiment is a repetition of Experiment II under different conditions. Ten loops of food were suspended in 10 cc. of a culture of *Colpoda duodenaria* with 900,000 free-swimming organisms per cc. Five cc. of this mixture were put into each of two test tubes, A and B. A was continuously aerated with a stream of small air bubbles, which

also effected uniform distribution of food and protozoa throughout the medium. B was not aerated nor shaken during the experiment.

Test tube A: In A the population increased, and the medium became nearly clear of bacteria. Food was added at intervals. After 48 hours A had a concentration of 2,480,000 protozoa per cc. After this the population began to decrease, probably due to excessive concentration of bacteria in spite of aeration, until at 72 hours it was 1,860,000 per cc. of very small *Colpoda*. Evaporation and some loss by frothing had reduced the medium to about 4 cc. Then four loops of food were added at one time. The result was the rapid death of almost all the protozoa.

Twenty-five loops of bacteria had been added by that time, so that the accumulation of metabolites from protozoa and bacteria may have intensified the lethal effect of the great concentration of living bacteria. There were no cysts of any kind.

To determine whether the metabolites were one of the lethal agents, and whether in spite of the metabolites the culture in A could be restored by aeration, at 98 hours 0,3 cc. were poured into a large shallow watch glass. The surface exposed to the air was therefore very large in comparison with the depth of the liquid.

At 120 hours the concentration of protozoa in test tube A was 248 per cc. In the watch glass the bacteria had collected into the center; the edges were nearly clear and here the protozoa had increased. At 149 hours all the *Colpoda* in test tube A were dead; there were no resting cysts. Three days later the medium in the watch glass was almost clear of visible food, the concentration of protozoa was enormous. Twenty-four hours later they had nearly all encysted.

This indicates that the lethal effect of a dense concentration of these bacteria on *Colpoda duodenaria* is due primarily to lack of oxygen, and that under the conditions of this experiment the metabolites neither prevent division nor induce encystment.

Test tube B: Two hours after the beginning of the experiment the concentration in B had dropped from 900,000 to 24,800 per cc. of sluggish protozoa, many of them evidently moribund. The liquid was hardly disturbed by the removal of a few drops for counting. After 24 hours a thin clear layer was visible at the surface. In it the concentration was 43,400 per cc. After 48 hours this layer had increased to $^1/_4$ inch in depth, and the concentration in it was 62,000 per cc. In the cloudy medium below it there were very few

ciliates. At 56 hours the concentration in the clear layer, $^1/_2$ inch deep, was 74,400 per cc. After this the population decreased. When the clear layer was about $^3/_4$ inch deep (50 % of the medium), it increased no further. At 150 hours the concentration was only 8,700 per cc. Food had become scarce in the clear layer, so there had appeared a ring of resting cysts on the glass at the surface of the liquid. There were no resting cysts anywhere else. There were also some small division cysts near the surface.

Experiment IV.

To determine the reaction of bacteria-free *Colpoda* in sterile medium, a number of these protozoa were washed once in the centrifuge and further washed by repeated transfers from medium to medium over a period of two hours with intervals of about 15 minutes in each dish. When one such bacteria-free *Colpoda* was placed in one drop (0.05 cc. is a relatively large amount) of sterile balanced medium, encystment invariably occurred in a few hours. The last series of ten such tests gave 80 % encystment in 3 hours, 100 % in 4 hours. There was no appreciable diminution in size during the pre-encystment period. These cysts spontaneously excysted without division.

Experiment V.

In order to determine whether sterile *Colpoda* in sterile yeast extract would encyst in the same way as in sterile balanced medium, some division cysts were washed several times in sterile balanced medium. The daughters were immediately removed and washed by repeated transfers in sterile medium. Finally one protozoon was put into one drop of sterile yeast extract in a petri dish. In all the cases the yeast extract remained clear until the end of the experiment.

These *Colpoda* made some growth, but very slowly. Eight of them formed small digenic division-cysts. The daughters made some growth, but only one divided again. During the first week no resting cysts were formed, but at intervals the free-swimming organisms encysted and excysted without division. During the second week the free-swimming forms decreased in size: some cysts of 12 μ diameter appeared. Apparently the medium used, namely, a 1 % solution of Difco Yeast Extract powder, affords sufficient nourishment to *Colpoda duodenaria* to maintain the free-swimming state and even to allow some growth for a time.

Experiment VI.

This experiment illustrates the trend of events when a few *Colpoda* are left in a relatively large amount of medium with a little food and a very little organic matter, on the breakdown products of which the bacteria can grow.

A quadrigenic division cyst was removed in a pipette from a growing culture; with it came a small amount of food and a corresponding quantity of bacterial and cyst-membrane debris. This cyst (diameter 30 μ) gave four progeny; two subsequent divisions produced 64 organisms. On the second day there were 63 free-swimming daughters, average length 25 μ, and one cyst, diameter 16 μ. On the third day there were 5 resting cysts, average diameter 15 μ; the length of the free-swimming ciliates varied from 20 to 35 μ. On the sixth day there were 24 cysts, diameter 9 to 16 μ, and 35 free-swimming forms, 12 μ to 20 μ long. Some cysts or free-swimming *Colpoda* had disappeared.

On the tenth day there were 17 cysts, the smallest of which was 7 μ across, and 25 free-swimming forms from 8 to 25 μ in length. Whereas the general tendency was downward in size of cysts and free-swimming forms, some of the latter suddenly increased in size to 25 μ, and some cysts, expecially the smaller ones, had disappeared.

On the twelfth day there were only 7 cysts, mostly the larger ones, and 21 free-swimming organisms. The smallest of the latter was 7 μ in length, but most of them were 16 to 25 μ.

On the fourteenth day there were only 4 free-swimming forms, of length 8 μ to 20 μ; and 24 cysts of which the smallest was 6 μ, the next 7 μ, and the others 11 μ to 15 μ in diameter.

It seems that there is a decrease in size of the free-swimming protozoa due to use of the substance of the body to supplement the waning supply of food obtained from the medium, and also to the production and loss of membranes in the recurring encystment and excystment process. Possibly the decrease in number of the small cysts after the seventh day and the increase in size of some of the free-swimming forms is due to cannibalism, but this has not yet been confirmed by actual observation. It might also be due to the discovery and consumption by some of the protozoa of small clumps of Pseudomonads growing on the remains of the very small *Colpoda* or their cysts which have died of exhaustion.

Discussion.

From the results obtained from this culture of *Colpoda duodenaria* in the same organic medium over a period of 16 weeks, it can be seen that the food concentration is a determining factor for the formation of cysts. The number of protozoa produced per loop of food is approximately constant. This is in agreement with PHELPS (1936) on *Glaucoma pyriformis*. All the growth and encystment curves are approximately parallel, and rapid encystment accompanies near-exhaustion of the food. Whenever the food concentration is raised by the addition of bacteria above a certain limit, encystment ceases. For instance, when 2 loops of food were added during the course of the encystment curve of Peak 9, encystment ceased, and the remaining non-encysted *Colpoda* began to feed and increase to Peak 10, with a maximum at about 16,000 per cc., which is also an increase of 5,000 animals per cc. per loop. It may be supposed that these remaining free-swimming *Colpoda* would all have encysted in due course if no food had been added, or that an equilibrium would have been established between the concentration of free-swimming protozoa and the food, if any, produced by growth of bacteria on debris.

The descending curve of Peak 10 shows that encystment, though at first as rapid as from the earlier peaks, occurs more slowly at the bottom of the curve. This slower rate of encystment seems to be due to the re-establishment of a favorable food concentration due to growth of bacteria on autolyzing debris. This feature, which becomes more evident as time goes on, is examined more closely at the bottom of Peak 12.

No food was given for 150 hours. When food became insufficient to maintain the dense population at Peak 12, all the animals would receive the impulse to encyst. While the encystment process was going on, the remaining bacteria, feeding on the cytolytic products of division cyst membranes, possibly some dead cysts and protozoa, etc., increase by division, until a favorable food concentration is re-established. Since all the free-swimming protozoa have received the impetus to encyst and since this influence has been operating for several hours, it is highly probable that the encystment would overshoot the mark, thus further increasing the favorable margin of available food. The remaining free-swimming protozoa will then increase by division until an insufficiency of food induces renewal of encystment. This also slightly overshoots the mark (at 1,200 hours);

then at 1,250 hours encystment is nearly complete (17 animals per cc.). There is almost certainly a succession of such steps at the bottom of the encystment curves. The accumulation of debris in the medium in the course of time accentuates this phenomenon.

An attempt was made in the twelfth week of the experiment to induce complete encystment from Peak 17 at 1,965 hours with a maximum of about 28,000 per cc. (not shown in Fig. 1). No food was added, encystment was precipitate down to a concentration of about 3,000 per cc. at 1,980 hours. From then on, it took 220 hours to bring the concentration down to 285 per cc. In the ninth week of a repetition of the experiment herein described a peak of 10,000 protozoa per cc. encysted rapidly to a concentration of 2,500 per cc., from then on with increasing slowness until it reached less than one *Colpoda* per cc. after 200 hours.

If we call R the amount of energy derived in a unit of time from capture and assimilation of food in the medium, then R will be a function of and will increase with the food concentration up to a limiting concentration. This limit may be determined by the physique or the physiological reactions of the protozoa, or by the toxic effect on them of high concentrations of bacteria.

We will consider here only food — concentrations up to the optimum, and within this range we may assume that R will increase with, but not necessarily be directly proportional to, the food concentration.

If we call R_e the energy requirement per unit of time necessary to maintain the fully differentiated (free-swimming) state, then a value of R greater than R_e, due to greater ease of capture, will result in growth and increase in number of organisms. A value of R less than R_e may be sufficient, with a contribution from the substance of the body, to maintain the free-swimming state, but at the expense of body size. The animals will and do, if R is not too low, become smaller and smaller (Exper. VI), but remain active and eat as much as they can capture.

It seems possible that there is a limit-value of food concentration below which the energy obtainable from the food of the medium, in addition to that obtainable from the organism itself, is insufficient to maintain the free-swimming state. It may be that, as a consequence, the animal subsides to the degree of dedifferentiation and inactivity of the encysted form.

We have seen (Exper. IV) that when the food concentration is zero, encystment occurs within a few hours. This pre-encystment

period will vary according to the amount of unassimilated food in the body, and during a part or the whole of this period the steps preliminary to the formation of a cyst will take place.

Experiment V shows that one *Colpoda* can obtain from one drop of yeast extract as prepared for this test, sufficient nourishment from the liquid and from particles suspended in it, to maintain the free-swimming state for at least two weeks. This amount of yeast extract allowed of some growth at first. In time the available food, perhaps the particles in suspension (OEHLER, 1919), were used up, and the organism decreased in size. This period of decrease in size was punctuated at intervals by encystment, followed at once by reorganization and excystment without division. It is doubtful whether the cysts ultimately formed should be considered true resting cysts. This question is receiving further study.

It is also possible to imagine a concentration of *Colpoda* so great that factors such as physical interference, insufficiency of O_2, etc., might reduce the rate of capture of food below the encystment-limit, however favorable the food concentration in the medium. This excessive concentration of the protozoa is greater than six millions to the cubic centimeter, and has not yet been experimentally obtained (Exper. II).

Experiment III indicates that a very high concentration of living food-bacteria so depletes the supply of oxygen that the protozoa die, but there is no formation of resting cysts. Experiment III B shows that when the medium is not disturbed by aeration the protozoa can only live at the surface where sufficient oxygen is available. They gradually increase in number and eat their way downward into the concentrated bacteria below. The increase in the clear layer is perhaps accelerated by the action of gravity on the suspended bacteria.

At a certain depth below the surface this process ceases, presumably because the diffusion down from the surface is not sufficient to maintain the requisite oxygen tension. The increase in population is, of course, slow because the large majority of precystic *Colpoda* sink to the bottom to encyst, and these would all be asphyxiated. Only those which adhere to the glass in the clear upper layer of the medium will be able to encyst and divide successfully.

Similarly, when the food in the clear layer decreases below the required minimum concentration, only those organisms which adhere to the glass near the surface will succeed in forming resting cysts. The others will die as they go toward the bottom of the tube.

Therefore neither lack of oxygen nor accumulation of metabolites is a direct inducer of encystment.

If now we consider the factors which have been suggested as inducers of encystment, we arrive, in the case of *Colpoda duodenaria*, in a well aerated unbuffered inorganic medium, at constant temperature (20° C.) and without evaporation or significant loss of medium, at the following conclusions:

1. Food in insufficient qnantity is the primary factor which induces the formation of cysts. In smaller quantities of medium it is easily demonstrated that food in large excess distributed in the medium causes decadence and even death in the culture, due mainly to oxygen deficiency, but never encystment (Exper. III).

2. Excretion products, accumulating in the medium during nearly four months, do not affect the growth and encystment curves. The response to food is similar throughout this time. The total number of protozoa which have grown and encysted is approximately 325,000,000 and to their metabolites must be added those of 130 loops of bacteria. It is possible that there may be some volatile metabolite of alkaline reaction which may be neutralized and fixed in the medium by the continuous supply of CO_2, or that some such volatile products may be carried away by the excess air and CO_2, but in any case it is evident that neither volatile nor non-volatile metabolites have had any influence on the occurrence of encystment.

3. Desiccation of the medium and consequent increase in salt concentration are not requisite factors for encystment because the medium did not decrease, except by the total of drops required to make the counts of population. It is quite possible that desiccation of a few drops of medium might indirectly induce encystment by crowding the animals into less medium with less food until the food supply is insufficient to maintain the free-swimming state. Such salts as have been added in the substance of the bacteria have been taken up by the accumulation of resting cysts on the glass.

4. Lack of O_2 in an aeration flask could not be a factor inducing encystment. This is evident also from consideration of Peaks 2 and 13; when the food is exhausted, encystment occurs just as readily in a population of 6,000 animals per cc. as in one of 115,000 per cc. It is easily demonstrated that if *Colpoda* are put into well-boiled balanced medium from the surface of which air is excluded by an adequate cover, the animals die without encysting. This does not exclude the possibility that formation of cysts may be to some extent induced by a decrease in the oxygen tension (Adolph, 1929;

RHUMBLER, 1888), but the direct factor is probably the inadequate supply of energy obtainable from the food in the environment under those conditions.

5. Variation of the temperature is not necessary to induce encystment, since the temperature remained constant at 20° C. throughout the experiment. Here, too, it is possible that high or low temperatures, by reducing the amount of energy obtainable per unit of time from the food of the medium, might indirectly induce some encystment.

6. Age of culture over a period of nearly four months does not affect encystment.

7. There is no sign of physiological periodicity in the growth and encystment of this ciliate.

8. p_H is not an inducing factor of encystment. The medium was not artificially buffered. The p_H of the original medium in equilibrium with the CO_2 of the air was 5.7; at the end of the first month it had risen to p_H 8; from then onwards it rose to and still fluctuates around 8.2. Growth and encystment occur as readily at p_H 8.2 as at p_H 6. At the peak of the curves there is a fall of a few tenths of a p_H unit due to temporary accumulation of CO_2 (see Peak 13).

9. Crowding of these protozoa does not, per se, induce encystment (Exper. II). A single specimen of *Colpoda duodenaria*, of any size in any quantity of medium, will always form a cyst if food is removed from the medium. There is, however, strong evidence (from unfinished studies) that crowding is an important factor influencing the permanence of induced cysts of this animal (cf. also BARKER and TAYLOR, 1931).

The importance of the food factor has been noted by several investigators. ADOLPH (1929) obtained resting cysts of *Colpoda* (species unspecified) in low concentrations of food. WEYER (1930) found that encystment of *Gastrostyla steinii* was induced only by hunger. BEERS (1927) says that lack of food is one of the factors which induces encystment of part of a culture of *Didinium nasutum*; the remainder of the culture dies of starvation. If a few *Colpoda* with a very small quantity of food are placed in a drop of medium, the rate at which they can derive energy from the food may be insufficient to maintain the free-swimming state. If, however, they can make up the deficiency by drawing on the substance of their own bodies, they will and do become smaller and smaller, though finally some, possibly all, encyst (Exper. VI). Cysts as small as 6 μ in diameter, free-swimming animals 8 μ in length, have been

noted, but it has not yet been possible to find as many of the small cysts as there were animals in the medium. It is the very smallest cysts that seem to disappear, and there are indications pointing to a correlated increase in size in some of the remaining protozoa.

The statement made above, that sterile *Colpoda* in sterile medium encyst within a few hours, is nevertheless invariably true. The protozoa used for the tests in sterile medium were taken at random from growing cultures and so were not of the same age and size, though large animals were discarded. The variation in the time required for encystment may well be due to the variation in the quantity of undigested food in them. Encystment thus induced is not necessarily permanent.

Penn (1934) states that deficiency of food in the medium is one of the factors inducing encystment in *Pleurotricha lanceolata*, and that organisms containing little food in the body encyst more readily than those containing much. He further says that the amount of food in the medium is an environmental, the amount in the body a physiological factor. It seems more reasonable to consider at least undigested food as equivalent in kind and effect to food in the medium and therefore an environmental factor. In the above-mentioned instance of *Colpoda* in sterile medium, only when the ingested food is assimilated would the absence of food in the medium have the effect of inducing encystment.

Summary.

1. *Colpoda duodenaria* was cultured for 4 months in an aeration flask containing 500 cc. of an inorganic balanced salt solution. The only additions to the culture were food-bacteria as required, the only subtractions the few drops necessary to make periodic counts of the population.

2. The formation of resting cysts accompanies the exhaustion of the food; the food concentration is an essential factor directly governing encystment.

3. Other possible factors, such as excretion products, desiccation of the medium, lack of oxygen, age of culture, physiological periodicity and p_H do not influence encystment under the conditions of this experiment.

4. The number of protozoa produced by the addition of a given quantity of food remains constant during the whole period.

5. The p_H of the unbuffered medium rose from p_H 5.7 at the beginning to p_H 8 after 8 weeks, and remained approximately constant thereafter until the end.

6. Bacteria-free *Colpoda* in a relatively large amount of sterile medium encyst in a few hours. The amount of undigested food in the animal may affect the interval preceding encystment.

7. A culture of six million protozoa per cc. was obtained by feeding continously; there were no resting cysts but many division cysts at the final count.

8. A few protozoa in a relatively large amount of medium with little food decrease in size, with periodic encystment and excystment.

9. The lethal effect of an intense concentration of the bacteria used as food in these experiments is due to the depletion of the oxygen supply below the minimum required for the existence of the protozoa.

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