Some Factors in the Excystment of dried Cysts of *Colpoda cucullus*.

By

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(With 3 figures in the text.)

Introduction.

For experimental studies on the living cell in its simplest dedifferentiated state, we have found artificially induced cysts of some ciliates to provide exceptionally good material. *Colpoda cucullus*, for example, may be caused to encyst at will under conditions which are easily duplicated. Such cysts will then withstand very excessive drying, high vacua and extreme temperatures, their tolerance to which can be readily determined by their proportion of inducible excystment. Although the inability to excyst may not be an absolute criterion of death, it seems reasonable to assume that an inability to redifferentiate under normally favorable conditions is a sign that the protoplasm is at least moribund.

The validity of this criterion obviously depends upon an analysis of the factors of excystment, involving both qualitative data and accurately quantitative measurements of excystment under specific and reproducible circumstances. To this end the following studies were undertaken.

Materials and Methods.

Colpoda cucullus is an excellent ciliate for experimentation. It is readily cultured in balanced medium, without any organic solutes, and it multiplies rapidly on a suspension of a pure culture of bacteria such as *Pseudomonas fluorescens* which is especially suitable for the purpose. This ciliate can be caused to encyst at will by crowding the culture (Barker and Taylor, 1931) and to excyst usually 100

percent in a suitable medium. Most extracts of vegetable or animal tissues within a wide range of concentration will induce excystment of *Colpoda* (Barker and Taylor, 1933), but to be sure of comparable results the same fairly reproducible medium must be used. The excystment medium finally selected and employed in all tests was made by dissolving 1 g. yeast extract paste in 1 liter of balanced salt solution. This medium can be autoclaved and duplicated at will, and it gives excellent results. The balanced salt solution (0.012 %) was after Osterhout:

H_2O	1000	cc.
NaCl	.104	g.
$MgSO_4$	.004	„
$MgCl_2$	.0085	„
KCl	.0023	„
$CaCl_2$	.001	„

As possible substrates, the following materials were tried: Glass (several kinds), quartz, mica, agar films, gelatin films, collodion films, waxed papers and several kinds of celluloid and Cellophane.

The material finally selected as admirably suited to the purpose was the Cellophane No. 600 P. T. (plain transparent, non-moisture-proofed, thickness 0.0017-inch), which was kindly supplied by the Du Pont Cellophane Company, Inc. of New York.

The method of inducing encystment was to crowd the culture (Barker and Taylor, 1931) by sedimentation in the centrifuge at a speed of about 1.000 r. p. m. Open glass vessels and flakes of mica were inoculated with the crowded culture by means of a micropipette which deposited drops of about $^1/_{50}$ cc. containing usually a few hundred protozoa. In this crowded condition, these protozoa begin encystment almost immediately.

Before testing for excystment, superfluous cysts, especially those not easily counted, were loosened with a needle and removed. Tests with preparations thus made and with untouched clusters of cysts show that the viability is not affected thereby.

Agar, gelatin and collodion films were made by drawing strong solutions into drawn glass tubes of 1 to 2 mm. diameter. The solution was then blown out, which left a film of the solution on the glass. The procedure can be repeated until the film is thick enough. When the film is sufficiently dry, a crowded culture of protozoa is drawn into the tube and allowed to encyst. The cysts can be counted by rotating the tube under the microscope. Convenient lengths of the

tube with sufficient countable cysts can be cut off for excystment tests. Controls were used to prove the viability of cysts before drying

The method of preparing the cysts on Cellophane was as follows. The protozoa were cultured in the afore-mentioned balanced medium. They were fed with a suspension of a pure culture of *Pseudomonas fluorescens*. When the culture became sufficiently concentrated, the protozoa readily encysted. Before this saturation point was reached the culture was centrifuged at low speed until the protozoa and debris were sedimented. A glass rod with a bulb at one end was introduced into the centrifuge tube; it cut off the protozoa in about $^1/_2$ cc. medium at the bottom of the tube. The medium above the bulb was poured off, about 5 cc. sterile medium added, and the bulb carefully removed. The protozoa dispersed into the liquid, which was then carefully decanted into a clean tube. This tube was filled with sterile medium and the process repeated five times. The final $^1/_2$ cc. of concentrated protozoa was then ready for use. Better results were obtained if two sets of tubes were used and the final sediments mixed. Tubes and medium should be sterilized before use. This method does not give an absolutely sterile culture but with care the number of bacteria present is extremely small and, for immediate purposes, negligible.

The Cellophane, after water-extraction, was laid on the larger half of a Petri dish, with an overlap equal, at least, to the depth of the dish. The smaller half was pressed into the larger half and the whole sterilized in the autoclave. For a 7 cm. Petri dish the concentrated suspension of protozoa was diluted to 5 or 6 cc. in order to cover the Cellophane with an even layer of medium. This diluted suspension was poured into the smaller half of the dish, the larger half with the Cellophane was pressed down into place, the whole was firmly held together with strong elastic bands, and then inverted.

The protozoa encysted rapidly. In about $1—^1/_2$ to 3 hours' time, depending on the rate of encystment, it was found advisable to invert the dish. The following day the medium was poured off and the cover and Cellophane rather loosely replaced. The Cellophane became practically dry in about 24 hours. The drying at room temperature and humidity was completed by placing the cover with the Cellophane on a piece of filter paper.

For purposes of experimentation this thoroughly dried Cellophane was carefully cut with a small sharp blade into small pieces containing clusters of cysts. In most instances, the number of cysts in a given cluster could be accurately counted in the dry state or, more easily, when immersed in the excystment medium.

An alternative technique, when skillfully executed, provided more satisfactory material for experimental work as follows: A device was constructed to make parallel grooves in the Cellophane. The grooves were approximately .25 mm. apart and of a cross-section which would just accommodate a cyst. Practically all of the protozoa encyst in these grooves in continuous rows, so that counting was rapid and accurate.

Experimental.

I. Substrates.

The suitability of various substrates was tested and the results are detailed below.

a) Glass: Attempts to induce excystment of *Colpoda* cysts air-dried on glass by addition of excystment medium were only moderately successful. Several kinds of glass were tried with similar results. It made no difference whether the balanced salt solution (with no organic content) was allowed to evaporate, leaving the cysts with a noticeable covering of salts or was removed as for as possible before dessication. The following typical test (Table 1) shows that under the conditions of these experiments, the cysts of *Colpoda cucullus* did not live long when dried on a Syracuse watch glass:

Table 1.
Viability of Cysts Dried on Glass.

1st day	Inoculation and encystment
2—5th day	Gradual dessication in moist chamber
6th „	Dry at room temperature and humidity
7th „	Excystment 12 percent
8th „	„ 4 „
9th „	„ 0 „
10, 11, 12th day	„ 0 „

The rate of drying was varied by changing the opening in the moist chamber, but the results were not affected thereby.

b) Quartz and Mica: The value of quartz and mica as substrates for dried cysts was no greater than that of glass, as illustrated in the following table.

Table 2.
Quartz Capillary Tubes.

Days after drying	7	7	10	10	15	15	22	22
Excystment %	12	16	10	0	35	0	0	0

Excystment from mica was in all cases nil. It is of interest to note that cysts dried on mica frequently swelled up and burst soon after immersion in the excystment medium.

c) Collodion, agar, gelatin, films, etc.: None of these was found satisfactory. Films of even thickness were difficult to make, to dessicate and to handle. Excystment was variable; swelling of the film on immersion in the excystment medium was a detriment; partial solution or excessive hydration of the film was liable to smother the cysts. The following tables (3 and 4) are representative of the results.

Table 3.
Agar.

Days after drying	2	6	6	8	10	18	18
Excystment %	100	5	0	0	0	0	0

Table 4.
Gelatin.

Days after drying	2	4	6	6	8	18
Excystment %	40	0	5	0	0	0

In tables 3 and 4 the word "drying" implies merely the disappearing of moisture under the conditions imposed.

d) Celluloid: Different samples gave very different results: some consistently nil. The following table shows the excystment obtained from the best sample.

Table 5.
Celluloid.

Days dry	2	4	5	11	13	16	30	50
Excystment %	60	88	80	50	25	5	7	0

Whether there is any correlation between the excystment obtained from different samples of celluloid and the camphor content of the sample has not been determined. The excystment was in most cases small.

e) Cellophane: In order to determine whether any treatment of the commercially prepared Cellophane, known to contain glycerine (as a softener) and perhaps other residues, might render it more suitable as an encystment substrate, selected Cellophane samples were subjected (1) to water extraction or (2) to heat under vacuum. For removal of the glycerine by water extraction, the samples were washed in a relatively large volume of triple distilled water, heated to 70° C. for several hours, and for the removal of possibly non-soluble, volatile inclusions, other samples were exposed to high vacuum (1×10^{-5} atmosphere) and a temperature of 150° C. for three hours. Comparative excystment figures, following the use of Cellophane as substrate after the treatments just noted, are shown in Table 6.

Table 6.
Treatment of the Cellophane before Inoculation.

Preparation Nr.	Treatment	Excystment %	
		34 days	61 days after drying
371	Water extracted, heated and evacuated	74	79
372	Untreated	72	69
373	Heated and evacuated	75	73

The variations are not sufficiently large or regular to indicate any improvement due to heating and evacuation.

II. Uniform size of cysts.

In the description of the technique of preparing the cyst-Cellophane material, it was stated that encystment should not be allowed to continue for more than three hours, i. e. until all the fully grown protozoa had encysted. In our earlier preparations this precautions was not taken, with the result that some of the largest cysts soon underwent binary division and excysted. The resulting young protozoa subsequently encysted in a semi-

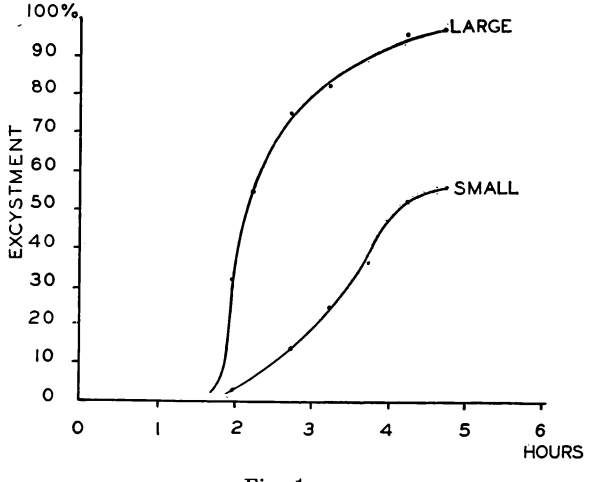

Fig. 1.
Viability and rate of excystment of large and small cysts.

Table 7.
Viability and Excystment Rates of Large and Small Cysts.

Time Hrs.	% excysted		Rate of excystment of viable cysts	
	Large	Small	Large	Small
2.0	32	3.6	33	6.3
2.25	55	11.6	56.7	20.6
2.75	75.4	14.3	77.6	25.3
3.25	82.6	25.0	85.0	44.4
3.75	87	36.6	89.5	65.0
4.25	96	52.7	98.5	93.6
4.75	97.1	56.2	100.	100.

starved condition, forming distinct rings of quite small cysts around the normal-sized cysts in each cluster. These small cysts are less viable than the full-sized ones, so that excystment resulting from such a preparation is not uniform.

Table 7 and figure 1 show (a) the relative viability and (b) rates of excystment of the large and small cysts.

III. Viability of cysts dried on Cellophane.

Figure 2 shows the variations in the excystment obtained from six pieces cut at random from cyst-Cellophane preparation No. 707, on the 17 th day after drying.

We have found that by careful attention to technique cyst-Cellophane preparations can be made such that samples cut at random from them will give excystment usually differing from the mean by less than 5 percent.

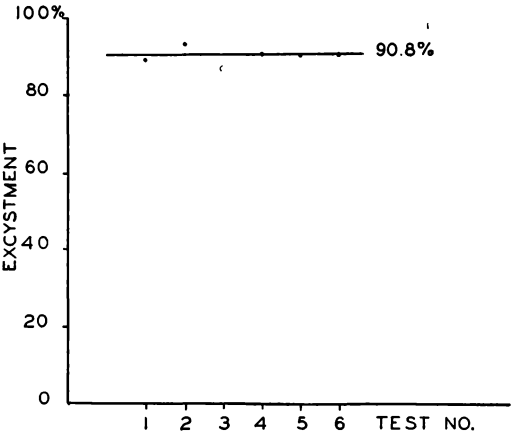

Fig. 2. Excystment of cyst-Cellophane preparation No. 707; 17 days dry.

IV. Decrease of viability with time.

In order to determine to what extent the excystment obtainable from a preparation of cysts on Cellophane varies with time, preparation No. 707 was kept in a Petri dish on a table in the laboratory

Table 8.
Decrease of Viability with Time.

No. of cysts Excystment			No. of cysts Excystment		
Days dry	tested	%	Days dry	tested	%
11	563	78.6	73	999	83.7
17	912	90.8	191	1279	71
24	1153	95	207	426	73
31	1099	88	214	501	78
38	1415	84.8	228	471	70
45	680	93.2	235	445	77.5
52	817	89	249	321	71.5
59	1625	88.2	262	218	74.4
66	1446	78	275	251	80

and pieces containing a convenient number of cysts were cut from it at intervals and tested for excystment. Table 8 and Fig. 3 show that the viability decreased about 15 percent in the first six months. From the sixt to the ninth month it remained fairly steady around 75 percent.

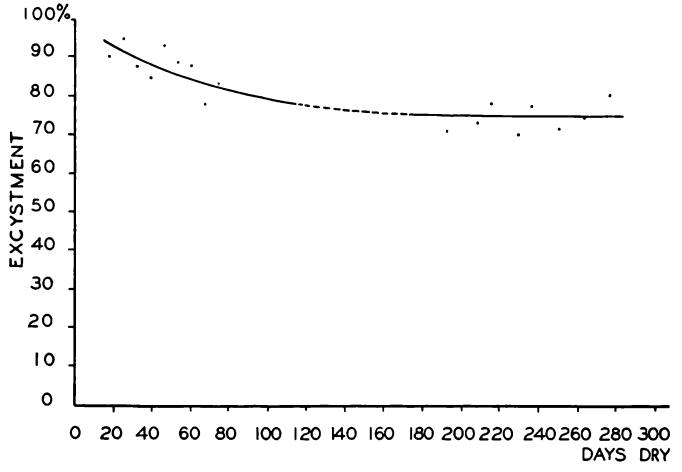

Fig. 3. Excystment of the dried cysts on Cellophane of Colpoda cucullus.

Unfortunately it was not possible to make excystment tests between the 74 th and 190 th day.

Discussion.

The ciliate *Colpoda cucullus* is a common, hardy, easily cultivated animal. Under certain conditions it forms a resting cyst within which no formed structure is visible except the nucleus. Encystment and excystment can easily be induced. It offers, therefore, a convenient material for experimental work on protoplasm in a relatively simple state.

It is obvious that, for quantitative experimental purposes, the substrate should be transparent in order to facilitate an accurate count of the cysts under test, and that is should, moreover, be absolutely innocuous to the encysted protozoa. It is not easy to understand why glass, mica and quartz are so unsuitable as a substrate for dry cysts, especially since the two last are so ubiquitous in the natural environment. This can hardly be due to toxic solutions from the substrate because the cysts remain viable for long periods in a minimum of medium in a glass container. These three materials

being almost impervious to moisture, it might be supposed that the rate or the degree of dessication might have some injurious effect.

Experiments in the moist chamber, as presented in the foregoing paragraphs, show that, within the range in question, the rate of dessication does not affect the percentage of excystment. That the degree of dessication is also relatively immaterial is proved by drying a cyst-Cellophane preparation in the oven at 90° C. for one hour, or in a high vacuum for several days. In neither case is the viability perceptibly lowered.

WEYER (1930) found that the cysts of *Gastrostyla steinii*, when all impurities were removed by washing in clean salt solution, were all dead 24 hours after drying. When dried in a mixture of their solid metabolic products, agar and bacteria, excystment of 80 percent was obtained after 18 days.

ILOWAISKY (1926) says the cysts must be dried in the complete detritus of the medium, as happens under natural conditions; that washed and dried cysts (on glass), first at room temperature, then for a few days at 25° — 30° C., mostly die.

L. RHUMBLER (1888) found that he could not keep dry cysts of *Colpoda* more than three weeks, but he neither mentions the substrate (presumably glass) nor states whether or not they were washed or covered with detritus.

Such inconvenient substrates as collodion, agar, etc. were discarded as useless.

It is possible that the camphor in celluloid may have a toxic effect on the cysts. In any case, its inflammability renders it undesirable.

Cellophane, an almost pure regenerated cellulose (HYDEN, 1929), is an excellent substrate for experimental purposes. It is well to note, however, that its glycerine plasticiser, while altogether nontoxic to these protozoa, may induce their excystment. It is advisable, therefore, to subject the Cellophane to a thorough extraction with water before use, in order that this plasticiser may not interfere with results in experimental work.

Treatment of the Cellophane by heat under vacuum did not change the results obtained.

The importance of preventing the encystment of any but mature protozoa is evident from the comparative viability of large (97.1 percent) and small cysts (56.2 percent).

Summary.

1. Dried cysts of *Colpoda cucullus* have proved to be decidedly valuable material for quantitative biological experimentation.

2. The viability of *Colpoda* cysts, dried in the absence of debris or other protective substances, was determined for various transparent substrates.

3. These ciliates induced to encyst and then dried on glass, quartz or mica did not remain viable for more than a few days.

4. On celluloid and certain other organic substrates some cysts remained viable for periods up to 30 days.

5. Cysts of adequate size which had been induced to form and to dry on Cellophane have retained for an indefinite time their ability to excyst. Their viability decreased slowly. After the sixth month, the excystment appears to be stabilized around 75%.

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