CIX. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA*. PART II.

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In a previous paper [Cutler and Crump, 1923] an account of experiments on mass cultures of *Colpidium colpoda* was given; the present communication deals with similar experiments carried out on cultures containing one or more cells isolated into one cubic centimetre or less of culture solution. The objects of these experiments have been to discover whether the results obtained from cultures containing few organisms in small quantities of fluid are comparable with those in which numerous animals are inoculated into comparatively large amounts of liquid, 10 cc. or more. Also there have appeared a series of papers by Robertson [1921, 1922], describing new and interesting phenomena —allelocatalysis, etc.—in cultures of a ciliate *Enchelys farcinem*. These seemed to be of so fundamental a character that it was felt desirable to test whether they obtained with other species of ciliates.

Methods.

The medium used has been the synthetic one in which the experiments on mass cultures were conducted. The smaller cultures (*i.e.* less than 0·09 cc. in volume) were put up as follows in unruled counting chambers 0·1 mm. deep: the individual, or individuals, are transferred, by a capillary tube from the parent culture, with about 0·01 mm.^{3} of liquid, to the chamber. It has not, however, been possible to keep this quantity rigidly constant, owing to the small volume of the fluid used; the chamber is then covered with a thick cover slip, on which there is already a drop of new medium, freshly inoculated with *Sarcina* to provide an adequate food supply, and the two drops are allowed to mix. The final size of the drop may vary from 0·37 to 9·8 mm.^{3}, and the degree of dilution varies between 1 in 37 and 1 in 980; these dilution figures, however, are only an approximation owing to the difficulty of measuring the original minute drops containing the organisms with accuracy, and, for the same reason, the size of the drop is not of necessity an index of the degree of dilution. Before the cover slip is put on the chamber a ring of small drops of sterile medium are placed in the groove around the raised central part to discourage

evaporation from the culture itself. The drop size in any culture is measured by projecting an image of the chamber on to squared paper, where the outline of the drop is drawn; as the magnification and the depth of the liquid (0·1 mm.) are both known, by counting the squares enclosed by this outline, the volume can be calculated. Each culture has been measured in this way every day so that changes in volume due to evaporation or condensation are detected. The numbers of animals present are counted by projecting the image of the chamber on to a screen, using a 48 mm. lens, and adjusting the eye-piece and distance of the screen so that the final magnification is from 50 to 60 diameters. With practice numbers of animals up to 100 can be counted with a sufficient degree of accuracy.

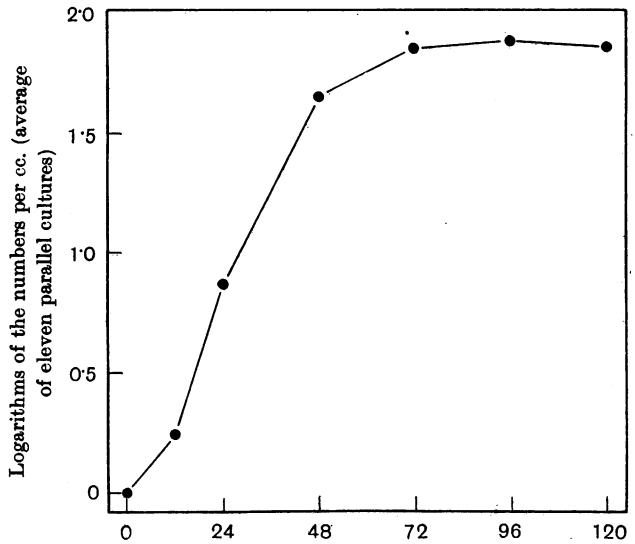

Fig. 1.

Results.

The curves obtained by plotting the numbers of animals in these single cell cultures against the time are similar to those derived from mass cultures, at least until the maximum numbers are reached; after this point, although some of the cultures show the same oscillations that occur in mass cultures, a certain number die out completely in 24 hours. Fig. 1 shows the curve obtained by averaging the numbers of animals every day in eleven parallel cultures (I series, 1 day parent), each starting from a single cell, and plotting the logarithms of these numbers against the age in hours (cf. Fig. 2 in previous paper). It will be seen that the sequence of events in these single animal cultures is in the main similar to that in mass cultures.

Allelocatalysis.

To test the allelocatalytic effect described by Robertson [1921, 1922] a number of experiments were made in which one, two, three or four animals were inoculated into drops of various sizes. The cultures were examined, usually after 12 hours' incubation, and then daily until discontinued. In Tables I and II are given the rates of reproduction arranged in order according to the size of the drop or to the equivalent number of animals inoculated per cc. of liquid. It will be seen that we have been able to find no evidence of the effect obtained by Robertson; for, according to his results, the reproductive rate should increase as the number of animals inoculated increases (Table I); and, the greater the initial concentration per cc., the greater the rate of reproduction should be (Table II). Nor does the size of the drop, within the limits of the experiment, appear to have any appreciable effect, though there is an indication that the animals in the larger drops (giving a low initial concentration) are capable of reproducing a little more rapidly than in the converse case. This would also appear to be in direct opposition to Robertson's theory of the necessity for the animal to develop a definite concentration of "X substance" before division can take place. It should be noted, however, that Robertson [1921] worked with a much larger drop than we have found possible (0·08 cc.) and when only one animal was inoculated the concentration would be only 12·5 per cc. This would materially affect the experiment, but, as *Enchelys farcinem*, the organism with which he worked, measures 50–70 μ long by 35–50 μ broad, while our variety of *Colpidium colpoda* is only about 45–55 $\mu \times$ 10–20 μ[1], the amount of protoplasm in a drop of the size that Robertson used and the amount in our large drops would not be markedly different. If, then, the quantity of "X substance" produced is at all proportional to the amount of protoplasm producing it, Robertson's results and ours are at variance. It is realised, however, that such an argument may be erroneous, but we would emphasize the fact that the "allelocatalytic effect" does not obtain with *Colpidium colpoda* isolated in drops of the varying sizes we have used. In his last paper, Robertson [1922] states that with *E. farcinem* "single individuals isolated into volumes exceeding 1 cc. very rarely survive and failures to reproduce on the part of individuals isolated into cultures exceeding 0·1 cc. in volume are not at all infrequent." This, as the author points out, is a common experience with micro-biologists.

Assuming that it is due to the inability of the organism, *E. farcinem*, to produce a sufficient concentration of the X substance to induce reproduction, a smaller animal such as *C. colpoda* ought to be incapable of division when isolated into 1 cc. of culture fluid. Experiments were therefore made to test this hypothesis. Here single animals were isolated into 0·25 cc. (4 per cc.), into 0·33 cc. (3 per cc.), into 0·5 cc. (2 per cc.) and into 1 cc. (1 per cc.); two animals were also isolated into 0·25 cc. (8 per cc.) and into 0·5 cc. (4 per cc.) of

[1] The sizes of the animals given in the footnote on p. 179 of our previous paper [Cutler and Crump, 1923] should be 30 $\mu \times$ 10·5 μ and 38·9 $\mu \times$ 21·6 μ respectively.

Table I. *Table showing the reproductive rates of* 1, 2, 3 *and* 4 *animals during the first* 24 *hours after inoculation in drops of varying sizes.*

Size of drop in cubic mm.	1 animal inoculated	Av.	2 animals inoculated	Av.	3 animals inoculated	Av.	4 animals inoculated	Av.
0–0·5	—	—	2·4, 2·7	2·55	—	—	—	—
0·5–1	2·0, 0·0, 1·0, 1·0, 2·0 2·0, 3·5	1·64	2·3, 0·7, 1·8, 2·5, 1·0 1·8, 2·1, 2·5, 3·2	1·99	1·6, 1·7, 3·2, 2·1, 2·4	2·20	1·7, 1·7, 3·3, 1·3	2·00
1–1·5	2·0, 2·0, 3·0, 2·0, 1·0 1·0, 2·0, 0·0, 3·9, 3·8 3·3, 2·0, 2·0	2·15	3·6, 2·3, 3·6, 3·9, 2·3 3·7, 2·5, 2·5	3·05	1·9, 2·0, 2·9, 2·7, 0·7 2·5, 3·8, 3·6	2·51	1·8, 3·3, 3·1	2·71
1·5–2	3·0, 4·0, 1·0, 2·0, 3·1 1·5, 1·0	2·23	1·0, 3·4, 2·5, 3·6	2·62	3·0, 2·8, 1·0, 2·9, 3·7 2·5, 3·4, 3·6, 2·1, 1·2 1·6, 2·6	2·53	2·6, 3·6, 2·0, 2·5	2·67
2–2·5	2·0, 3·9, 1·0, 1·0	1·97	3·8, 2·7, 2·0, 1·5, 1·3	2·26	2·2, 2·3, 3·4, 1·6, 1·9 1·6	2·16	3·3, 2·0, 2·2	2·83
2·5–3	3·0, 3·0	3·00	1·0, 3·4, 2·3, 1·3	2·00	1·4	1·40	1·5	1·50
3–3·5	3·0	3·00	—	—	2·9, 2·7, 4·1, 2·0	2·92	2·7, 3·5, 3·5, 1·3	2·75
3·5–4	1·5, 4·0, 4·4, 4·6, 3·8 2·0	3·38	—	—	2·3, 1·7	2·00	—	—
4–4·5	2·0, 3·0, 2·0, 3·0	2·50	—	—	4·1	4·10	1·7, 2·4	2·05
4·5–5	3·9, 1·0	2·45	3·0	3·00	—	—	2·8, 2·1	2·45
5–5·5	2·0, 2·8	2·40	4·5	4·50	1·0	1·00	—	—
5·5–6	2·0, 3·3, 3·1	2·80	—	—	1·4	1·40	—	—
6–6·5	—	—	2·1, 3·8	2·95	—	—	—	—
6·5–7	3·7, 1·5	2·60	—	—	—	—	—	—
7–7·5	—	—	4·3, 1·0	2·65	—	—	—	—
7·5–8	—	—	—	—	—	—	3·3	3·30
8–8·5	3·4	3·40	3·0	3·00	—	—	—	—
8·5–9	—	—	—	—	—	—	3·3	3·30
			Average for drops of all sizes					
	2·4		2·5		2·4		2·5	

Table II. *Table showing the reproductive rates during the first* 24 *hours in cultures whose initial concentrations vary from* 100 *to* 8000 *per cc.*

Number of animals per cc. inoculated	Reproductive rate for the first 24 hours	Av. rate of reproduction for the first 24 hours
100– 200	2·0, 3·9, 3·3, 2·0, 2·5, 3·1, 3·4, 2·5, 3·7	2·96
200– 300	2·5, 1·0, 1·0, 2·0, 3·9, 3·0, 1·0, 3·0, 3·0, 3·8, 2·0, 4·0, 4·0, 4·2, 4·5, 1·0, 2·0	2·70
300– 400	3·9, 3·0, 3·0, 2·1, 3·8, 4·5	3·39
400– 500	2·0, 2·0, 1·0, 3·3, 3·0, 1·0	2·05
500– 600	3·0, 0·0, 4·0, 2·5, 1·0, 3·3, 2·0, 1·0, 1·0, 1·4	1·92
600– 700	2·0, 1·8, 3·9, 3·1	2·70
700– 800	2·0, 3·0, 1·0, 1·5, 1·0, 2·3, 1·0, 2·0, 3·4, 4·1, 3·3, 2·3	2·23
800– 900	1·0, 2·0, 3·7, 2·9, 2·8, 2·7, 2·0, 2·4, 1·7, 2·0, 2·0, 2·5, 1·7	2·23
900–1000	1·0, 0·0, 1·0, 2·0, 1·0, 1·0, 3·8, 2·7, 1·7, 4·1, 2·0	1·84
1000–1100	3·5	3·50
1100–1200	2·5, 2·7, 1·4	2·20
1200–1300	3·3, 2·2, 3·6, 3·5, 3·6, 3·6, 3·4, 1·8, 1·5, 1·3	2·78
1300–1400	1·0, 2·0, 1·7, 1·6	1·57
1400–1500	2·0, 2·3	2·15
1500–1600	2·0, 1·0, 3·7, 2·3, 3·6, 2·5	2·51
1600–1700	1·0, 3·5, 2·5, 3·7, 3·3, 2·6	2·76
1700–1800	2·9, 2·1, 1·6	1·40
1800–1900	2·9, 2·1, 1·6, 1·0, 3·0	2·12
1900–2000	0·0, 2·5, 3·6, 2·0, 3·3, 3·8, 3·1, 2·1, 1·2, 2·5	2·41
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2000–3000	2·0, 2·6, 3·5, 1·0, 0·7, 2·5, 1·8, 1·9, 3·3, 3·1, 0·7, 2·1	2·10
3000–4000	2·4, 0·5, 2·3, 1·8, 1·0, 2·5, 3·1, 1·8, 2·1	1·94
4000–5000	3·0, 1·2, 2·7, 1·3	2·05
5000–6000	0·0, 2·4, 1·6, 1·6, 1·7	1·46
6000–7000	1·0	1·00
7000–8000	3·2	3·20

liquid. The medium used in these experiments was one in which 0·024 % of ammonium phosphate and 0·016 % of glycerol were substituted for the sodium phosphate, ammonium lactate and glucose of the medium used in the other experiments. Where only one animal is present any growth must be due to that animal, but where two are introduced there is always the possibility that one may have died. Attempts were made to find the animals immediately after inoculation, and also to count them by direct examination of a projected image of the culture tube; this can only be very unsatisfactory even in 0·25 cc. cultures, though by using small tubes (2 × 0·3 inches), and immersing them in water to lessen refraction, a satisfactory image can be obtained and a rough approximation to the number of animals made. When the numbers rise to 400 per cc. or more, counts are made in a counting chamber.

The following table shows the figures obtained.

Table III.

Initial no. per cc.	No. of parallels	Average reproductive rate
1	5	14·94
2	5	13·80
3	5	13·60
4	7	12·72
8	2	11·05

These results show that the organism is capable of vigorous reproduction even when isolated into relatively large volumes of liquid[1].

Acceleration due to X substance.

The experiments detailed above demonstrate that the action of the growth promoting substance X is not so simple as might be thought by a perusal of Robertson's communications; but the work of this author and of others leaves little doubt but that some kind of accelerative agent is produced during the growth of organisms in culture fluid.

Since the publication of Wildiers' paper [1901] on the growth of yeast cells, where it is shown that the yeast does not develop its optimum reproductive capacity in the absence of an unknown substance, termed by Wildiers "bios," many investigations have been made to determine the nature of the accelerative agent. This work need not be detailed here as already many summaries have been made. It may be recalled, however, that Cantani [1901] reports that the growth of *Bacillus influenzae* is enriched by the presence of *C. diphtheriae*, *M. gonorrhoea* and certain staphylococci; a result also obtained by the use of dead bacteria as the stimulating agent. Such observations have been confirmed and extended by Neisser [1903] and Thjötta [1921, 1, 2]. Very few observations have been made on organisms other than bacteria, though Haberlandt [1913, 1920] adduces evidence that the plant cell produces a substance that is capable of inducing rapid cell division.

In view of the importance of these conclusions we have performed similar

[1] That single bacteria are also able to grow when inoculated into relatively large volumes of medium has been shown by Barber [1908], Churchman and Kahn [1921], and others.

experiments on *Colpidium colpoda*. The results, given in Table IV, show that crushed *Sarcina* or crushed *Colpidia* have the power of accelerating cell division; though the action does not seem to occur during the first 24 hours of incubation. This is contrary to the findings of Robertson, and is difficult to understand on the analogy of enzyme action.

Also the possibility has not been excluded that the increased reproduction is due to an increased food supply afforded by the crushed fragments of protozoa or bacteria. Such a possibility, however, obtains also in Robertson's experiments.

It is our intention considerably to extend these experiments, but in view of the interest of the problem it seems worth while to record the few observations already made.

Table IV. *Showing the accelerative effect due to the presence of crushed* Sarcina *and crushed* Colpidium.

Reproductive rate at the end of 24 hours' growth.

Initial concentration	Control		Av.	Crushed *Colpidium*	Av.	Crushed *Sarcina*	Av.
100– 500	1·0		1·00	—	—	1·0, 1·0	1·00
500–1000	1·0	1·0, 0·0, 1·5, 1·0, 0·0	0·75	1·0, 2·0, 1·5, 1·0, 0·0, 1·7	1·20	0·1, 0·0	0·05
1000–1500	1·0		1·00	1·4, 1·0, 1·0	1·13	—	—
1500–2000	0·0		0·00	0·0	0·00	—	—
2000–2500	1·0		1·00	0·1	0·10	—	—
2500–3000	0·5		0·50	1·4	1·40	—	—
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5000–5500	0·0		0·00	—	—	—	—
6000–6500	1·0		1·00	—	—	—	—
8000–8500		—	—	1·0	1·00	—	—
Average:		0·69		1·00		0·52	

Reproductive rate after 48 hours' growth.

100– 500	2·0		2·00	—	—	4·8, 3·9	4·35
500–1000	1·0, 2·0, 2·0, 2·9, 2·0, 1·0		1·81	3·8, 4·5, 4·2, 1·8, 2·0, 3·3	3·26	3·7, 4·6	4·15
1000–1500	1·0		1·00	4·3, 1·0, 2·9	2·73	—	—
1500–2000	0·0		0·00	0·0	0·00	—	—
2000–2500	1·5		1·50	2·8	2·80	—	—
2500–3000	0·7		0·70	2·5	2·50	—	—
.....							
5000–5500	0·0		0·00	—	—	—	—
6000–6500	1·4		1·40	—	—	—	—
8000–8500		—	—	3·1	3·10	—	—
Average:		1·34		2·78		4·25	

In connection with the accelerative effect of extracts of *Colpidia* and *Sarcina* it is interesting to note the action produced by the inoculation of fluid from a culture which had grown well into one which had shown a low rate of reproduction. The organism on which this test was made was a flagellate, *Oicomonas termo*. On May 15th, 1922, there was inoculated into 20 cc. of ammonium phosphate + saccharose medium sufficient *Oicomonas* to give a concentration of 70,238 per cc. The culture (*Z* 2) during the first 24 hours decreased in numbers to 51,500 per cc., but at the end of 72 hours had increased to 2,575,000 per cc.; thus showing a reproductive rate of 5·6. Concurrently with this another culture, *X* 1, was started in the same amount of culture fluid but with a concentration of only 17,559 per cc. During the first few hours of incubation the numbers fell to 7000 per cc., and at the end of 72 hours had

reached 13,000, showing a reproductive rate of only 0·89. At this juncture, therefore, a portion of $Z2$ was centrifuged to eliminate the organisms, and 1 cc. of the supernatant fluid was added to $X1a$, a culture formed by dividing $X1$ into two equal 10 cc. portions.

Hours:	0	4	24	48	96	120	144
$X1$	13,000	9,500	2,000	2,000	under 1000	under 200	under 200
$X1a$	13,000	17,500	31,000	20,000 + cysts	21,000	15,000	2,000

From the above table it is seen that, while the control culture $X1$ steadily decreased in numbers, the treated $X1a$ culture, after 4 hours, exhibited the effect of the treatment, and after 24 hours had had a reproductive rate of 1·25.

The experiment is also of interest in demonstrating that the decrease in numbers in an old culture is not primarily conditioned by the culture fluid becoming toxic: for the numbers of $Z2$, whose culture fluid was added to form $X1a$, steadily fell after the beginning of the experiment. This conclusion has been independently arrived at by Robertson from other evidence, and was also put forward by us in our previous paper.

Rate to first maximum.

In our previous paper it was shown that, when the logarithms of the numbers inoculated into a constant volume of medium were plotted against the maximum numbers attained, a reproductive rate varying from five to six divisions was found for 91 % of the cases. This held for inocula of 100 to 700 per cc., for parent cultures from 24 to 48 hours old, and the time required for the divisions varied from 48 to 168 hours.

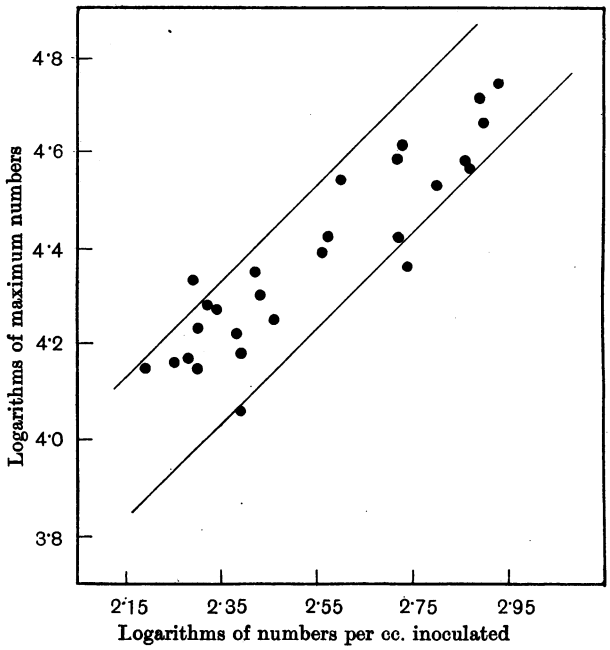

Fig. 2.

In the single cell experiments described above the majority of the cultures were not kept sufficiently long to attain their maximum numbers, but in 29 cases this was done. When the procedure adopted for the mass cultures is used it is found that again the reproductive rates fall within narrow limits, but instead of being from 5 to 6 divisions they are from 5·5 to 6·5 divisions (Fig. 2). This obtains, however, only when the initial concentration is equivalent to numbers lying between 100 and 900 per cc. Within these limits the age of the parent culture may be 24 or 48 hours, and the time taken to reach the maximum has varied from 72 to 168 hours. With concentrations above 900 per cc. the reproductive rates fall rapidly, as is shown in the following table:

Culture	Initial concentration per cc.	Rate	Final concentration per cc.
F 4	906	3·00	8,384
H 10	912	5·46	37,620
G 8	920	5·42	40,334
H 2	981	4·80	39,236
G 2	997	4·08	17,459
G 10	1,560	4·00	23,850
H 19	1,686	4·50	40,894
I 4	1,704	3·93	25,856
H 18	1,720	3·70	23,582
B 17	1,857	3·62	30,784
B 10	2,380	3·70	34,700
D 1	2,475	1·50	8,534
G 12	2,838	1·73	12,507
D 2	3,220	0·80	5,220
E 2	5,292	0·00	5,292
E 6	6,116	1·10	11,830

It was thought possible that the high concentration giving a low reproductive rate might be explained as follows: suppose each organism was capable of dividing 5 times, and, that, at a certain concentration x, autointoxication occurred; then, it might be, that when the initial concentration was high, the critical concentration x was obtained before 5 divisions had occurred. This, however, will not explain the result, since a culture *G*5, which had initially two animals and a concentration of 848 per cc., divided 5·9 times and attained a final concentration of 54,570 per cc. From the above table it will be seen that the final concentrations, in those cultures showing a depressed rate of reproduction, are below that of *G*5; though in the majority of cases if an extra division had taken place the concentration would have been above or near to that found in *G*5. Also with concentrations considerably below 100 per cc. the rate of reproduction does not fall within the limits 5·5–6·5, but is considerably increased as is shown in Table III.

These results indicate that with inocula of this size the animals tend to reach a constant maximum concentration instead of dividing a definite number of times as they seem to do when the inocula lie between 100 and 900 per cc. This tendency to arrive at a constant population is also seen when part of a mass culture is centrifuged to reduce the number of animals, and the supernatant fluid poured off. The reproductive rate of the animals left in this fluid is higher than it is in those that remain in the original culture.

SUMMARY.

The rate of reproduction of *Colpidium colpoda* has been tested in cultures derived from one or more animals isolated into small volumes of fluid. It is shown that in the main such cultures are comparable with mass cultures.

The allelocatalytic effect, described by Robertson, has been tested for and found not to obtain with *Colpidium* when isolated into fluid whose volume varies from 0·5 to 8·5 mm.^{3}. A few experiments are given in support of the contention that the rate of reproduction can be accelerated by the addition of small quantities of crushed bacteria or protozoa.

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