CXIX. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA*. PART III.

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The rate of growth and the number of divisions a unicellular organism undergoes in a given period of time has attracted the attention of biologists for many years. Numerous publications by bacteriologists have appeared on this subject; and the work of Maupas [1888], followed later by that of Woodruff, Calkins and others, has brought it into prominence as regards protozoa.

In 1923 we published the results of certain experiments on the ciliate *Colpidium colpoda*, our object being to show the necessity of keeping experimental conditions rigidly constant if comparable results were to be obtained. Among other variable factors the food supply was considered. It was realised that this was a factor particularly difficult to control, owing to the fact that the culture of the ciliate was contaminated by a species of a small bacillus. This was extremely difficult to exclude from the culture; and, moreover, when its numbers were reduced the ciliates showed typical signs of hunger degeneration, making it impossible to carry sub-culturing further. To obviate the difficulty, therefore, we decided to give to each sub-culture, either at the time of inoculation, or after 24 hours' growth, a plentiful supply of an easily recognisable species of bacterium, *Sarcina lutea*, in the belief that so long as plenty of these were present the food supply would be more than adequate to meet the requirements of the protozoa. Our experimental results appeared to justify this assumption, which also received support from the work of other observers, who had worked on a similar basis. Further, as the ciliate we were using was of the same strain as that used by Peters [1921], for his work on the growth of protozoa without bacteria, the possibility of deficient bacterial food supply seemed remote.

Recently, however, we have had to revise our views for the following reasons. Experiments were started to test which of the two bacteria, the contaminating bacillus or the added Sarcina, provided the better food supply. This involved counting the numbers of bacteria and protozoa in the cultures; whereupon it at once became evident that there was a very definite relationship between the number of divisions of the ciliate and the numbers of bacteria present.

The original experiment was therefore discontinued and new ones started. Here the procedure was to count the numbers of bacteria (the "contaminating" bacillus alone being used, because it seemed to be more acceptable to the ciliate than was Sarcina) and the numbers of protozoa at 24-hour intervals until the protozoa had attained their maximum numbers. In order to reduce the results to a common basis the bacterial numbers were expressed as a ratio, obtained by dividing the figure for the protozoa into that for the bacteria at each counting period. The ratio thus obtained gives the number of bacteria for each individual protozoan. In Table I the number of divisions for 24-hour periods are grouped together against the bacterial ratios which are also grouped as a geometrical progression. It is seen that the number of divisions increases as the number of bacteria per individual protozoan increases. When the ratio is as low as 500 little or no reproduction takes place, but at a ratio of 1,024,000 the number of divisions becomes as great as 5·3.

Table I.

Ratio	Reproductive rate for 24-hour periods	Average
250	0	0
250–500	0, 0, 0·2, 0·1, 0, 0, 0	0·04
1,000	0·1, 0·4, 0·04, 0·1, 0·03, 0, 0, 0	0·08
2,000	0·1, 0·2, 0, 0·6, 0·1, 0, 0, 0, 0	0·11
4,000	0, 0·1, 0, 0·2, 0, 0·6, 0·3, 0·8, 0·5, 0·4, 0, 0, 0·4, 0, 0, 0, 0·8, 0·6, 0, 0, 0·3, 0·2, 0, 0·4, 0, 0·6	0·24
8,000	0·4, 0·2, 1·4 0, 1·1, 0·7, 0·8, 0, 0·3, 0, 0·5, 0·6, 0, 0·6, 0, 0·2, 1·0, 0·4	0·45
16,000	2·5, 0·4, 1·5, 1·4, 1·3, 0·6, 1·0, 1·1, 0, 0·6, 0·7, 0·5, 0·2, 0, 0·1, 0·7, 0·6, 0·3, 0·9, 0·3, 0·3	0·71
32,000	1·1, 2·1, 0, 0·5, 3·3, 1·0, 0·5, 0, 0·3, 2·1, 2·0, 3·1, 1·2, 2·4, 2·4	1·47
64,000	1·8, 2·0, 1·9, 2·5, 2·9, 3·1, 3·1, 3·1, 2·9, 1·1, 2·7, 1·2, 3·3, 2·7	2·45
128,000	3·4, 3·2, 3·3, 3·4, 3·8, 3·3, 1·8, 2·9, 1·9, 2·3, 2·8, 3·1, 2·1, 2·9, 3·2, 2·7	2·88
256,000	3·2, 4·7, 4·2, 3·6, 4·0	3·94
512,000	4·0, 3·9, 3·6, 3·5, 4·8, 4·8	4·10
1,024,000	5·5, 5·5, 4·9	5·3

It should be pointed out that this is irrespective of the period of time which has elapsed since the culture was started; a culture that has been growing for only 48 hours will give approximately the same number of divisions as one that has been growing for 4 days, provided the ratio in the two cases is the same. For instance, one of our cultures in which, after 8 days, reproduction had ceased and the bacterial ratio was down to 640, was fed with the bacillus so as to raise the ratio to 12,400. Within the next 24 hours 0·9 division had occurred, though the unfed controls showed no reproduction. This phenomenon was repeatedly observed.

Further, it is found that in young cultures the number of divisions in the first 24 hours is dependent on the ratio as the following experiment shows. Cultures were started in which the number of Colpidia was constant, 115 per cc., but the bacterial numbers were varied so that the first series had a ratio of 34,000, and the second, 290,000; in the former the number of divisions was 1·89, but in the second 3·89.

On another occasion when two single cells had been inoculated each into 10 cc. of medium the cultures were fed with bacteria which were then counted,

giving an initial ratio of 18 millions in one case and 4 millions in the other. After 24 hours the numbers of Colpidia were still too low to be counted (under 100 per cc.), but after 48 hours the reproductive rates had been 12·0 and 11·5 respectively, rates which are very much higher than have been obtained, even in 48 hours, in any of the other cultures in which the bacterial ratios have been recorded (see Table I).

This intimate relationship between the bacterial numbers and the reproduction of protozoa probably explains the curious effect which an alteration in the protozoal population of a culture has on the subsequent division. Thus Robertson [1921] has shown that a culture in which reproduction has ceased, can be started again by removing some of the protozoa, the decrease in population being brought about by gently heating. This observation we have confirmed, using gentle centrifuging to remove some of the protozoa.

The most probable explanation of the phenomenon would seem to be that the mere decrease in protozoan population will cause an increase in the bacterial ratio. In the case of heat more ciliates will be killed than bacteria, because of the greater heat resisting power of the latter; while by slow centrifuging the ciliates are carried down, leaving the greater proportion of bacteria still suspended in the supernatant fluid.

Further, the increase in reproduction following inoculation into "bacterised fluid" (that is, culture media in which the bacteria have been allowed to multiply before inoculation with protozoa was made) finds a ready explanation in terms of the bacterial ratio. Robertson [1921] cites one case in which single individuals from two 8 day old cultures were isolated into bacterised fluid and within 24 hours produced 33 and 31 individuals respectively. This is comparable with our experiments, where after feeding with bacteria renewed reproduction took place.

This explanation of course applies only where the bacterial infusion has not been filtered or boiled; it does not therefore cover all Robertson's cases, though here there is the possibility that filtered bacterised fluid may stimulate the growth of bacteria, and so increase the ratio. It is quite possible that the existence of small quantities of X substance is necessary for high reproduction, as asserted by Robertson and others. But it is now evident that regard must also be paid to the action of such substances on the bacteria as well as on the protozoa: for a stimulation of the former will inevitably lead to an increased rate of the latter.

There are two striking differences between Peters' results with *Colpidium colpoda* and our results with the same strain. His cultures very seldom attain to such high numbers as do ours, in fact he considers that 10,000 or more per cc. constitutes an exceptional number of organisms, while ours seldom drop below this number and are generally considerably above it; and, further, this maximum is reached very much later, after about 12 days as against 6 days or less in our cultures. These discrepancies are readily explained on the supposition that Peters' cultures were contaminated with the same bacillus

that occurs in ours, but that he had reduced their numbers to a minimum. Such being the case the ratio would be very low from the beginning of every sub-culture, and the subsequent growth of the Colpidia would be slow, as the bacteria would have no opportunity to make headway and raise the ratio to a satisfactory figure.

Size of inoculum.

In previous papers it was stated that the variation in the number of animals inoculated, within limits (100–700), appeared to bear no relation to

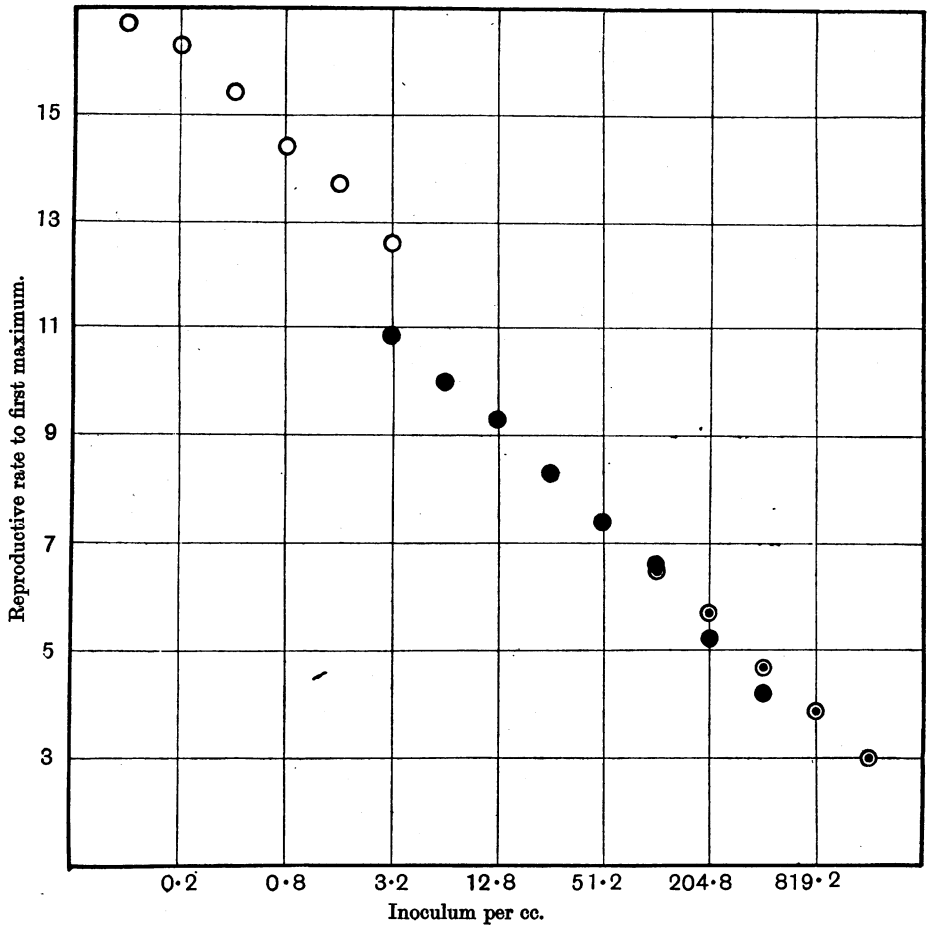

Fig. 1.

○ 1–3 animals in 0·25 to 10 cc. ● Mass cultures inoculated with 4–820 animals per cc.
⊙ 1–3 animals in less than 0·005 cc.

the subsequent growth of the culture, except in the few cases where single cells were inoculated into 0·25–1·0 cc. That is to say, that the reproductive rate is practically constant, lying between 5·0 and 6·5 divisions. Later work

has shown that this statement is misleading. If all the results are ranged in ascending order according to their inocula, beginning from one animal in 10 cc. and ending at 1600 per cc., and the inocula are then grouped in geometrical progression the reproductive rates fall steadily, the average for each group being greater approximately by one than that for the succeeding group (Fig. 1). The cultures whose results are shown in Fig. 1 were all grown in the same medium, and in the great majority of cases are derived from 1 or 2-day parents; they fall into three groups according to the method by which they were made. Those with the lower inocula, that is, from 0·1 to 4 per cc. (groups 0·1–0·2 to 3·2–6·4), came from single cells isolated into comparatively large quantities of medium, 10 cc. being the largest amount used and 0·25 cc. the smallest.

The second group of results shown in Fig. 1 come from mass cultures made in the ordinary way; that is, the number of cells in a stock culture are counted, and the amount of this culture that will give a suitable concentration in 10 cc. of fresh medium is calculated, and taken out in a sterile pipette. The inoculum in the mass cultures varies from 4 to about 500 per cc. Thus an inoculum of 4 or its equivalent per cc. occurs both in the first and second groups, in one case by the inoculation of one animal into 0·25 cc., in the other by inoculating 40 into 10 cc. The average reproductive rate is, however, different in the two cases, the single cell cultures giving a higher number than the mass cultures. It is possible that this discrepancy is due to the fact that in the mass cultures the number inoculated is not of necessity accurate, but it seems more likely that there is some real difference, as a line drawn through all the points of the first group is on a higher level than one drawn through those of the second, showing that the difference is common to all the points concerned.

The third group of results is derived from single cells grown in counting-chambers [Cutler and Crump, 1923]; here the inoculum is in every case a single cell, but, as the volume of liquid is very small, it is equivalent to a high concentration per cc. The points in this group fall very well into line with those of the second group.

The conclusion to which these results obviously point is that each culture tends to arrive at a constant maximum population per cc. irrespective of the number of animals from which it starts. That earlier work suggested a constant reproductive rate between certain limits was due to the fact that the limits were comparatively narrow and that relatively few results were available. In Table II all the results of Fig. 1 are given, together with the coefficient of variation and range of each group, and in this case all the cultures are massed together regardless of the way in which they were made.

As has been pointed out before [Cutler and Crump, 1923], Robertson [1922] found considerable difficulty in making successful cultures of *Enchelys* from single cells in 0·1 cc., or more in volume, and, further, stated that this was a common experience. A review of the literature bearing on this point, however, indicates that both bacteria and protozoa can be isolated into very

Table II.

Inoculum per cc.	Standard deviation	Range	Average reproductive rate	No. of cases
0·1–0·2	0·405	1·5	16·7	16
0·2–0·4	0·705	2·2	16·3	8
0·4–0·8	0·389	1·1	15·4	8
0·8–1·6	0·545	1·8	14·4	13
1·6–3·2	0·511	1·7	13·7	12
3·2–6·4	1·166	3·7	11·6	19
6·4–12·8	0·701	2·7	10·0	10
12·8–25·6	1·130	2·9	9·3	12
25·6–51·2	0·520	1·8	8·3	14
51·2–102·4	1·233	3·4	7·4	14
102·4–204·8	0·619	2·6	6·6	25
204·8–409·6	0·823	2·4	5·5	32
409·6–819·2	0·719	3·0	4·7	17
819·2–1638·4	1·334	4·2	3·9	10
1638·4–3276·8	1·229	3·7	3·0	8

much greater volumes without impairing their reproductive activity. Thus Barber [1908] has grown single bacteria in 5–10 cc. of broth and records that his failures are probably due to faulty technique; Churchman and Kahn [1921], working with *B. coli*, found that in 80 % of the cases single bacteria would grow, presumably in large quantities of broth, though the exact amounts are not stated. In the case of protozoa, Balbiani [1860] asserted that single Paramoecia require 2–3 cc. of infusion for the greatest reproduction to take place; Woodruff [1911] found that Paramoecia in 40 drops divided 7·4 % times more rapidly than those in 2 drops, and Taliaferro [1923] claims to have infected rats with single specimens of *Trypanosoma lewisi*.

The results obtained in our work bear out Barber's contention that failure of single cells to grow in large volumes of liquid is due to imperfect technique, and further indicate that speed in transferring the isolated animal from the parent culture to the new medium is the most important point to be observed.

From the point of view of the bacterial ratio the single cell cultures in 10 cc. of medium form an extreme case which should be of interest. In all, 16 of these cultures have been grown and in every case the parent from which the single cell came was 24 hours old. The first set contained four parallels and was unfed, the final average reproductive rate was 16·4, and the maximum number of Colpidia was attained on the 8th and 9th days after inoculation. There were six parallels in the second set, again unfed; here the average reproductive rate was 17·0 and the maximum numbers occurred on the 5th and 6th days. In all the other cases the cultures were fed with the bacterium; in four of these the average content of bacteria after 24 hours was 87 millions; in the other two, put up on another occasion, the average content after 24 hours was 180 millions. Of these six cultures, all reached their maxima on the 3rd or 4th days after inoculation, in the first case with an average reproductive rate of 16·9 and in the second of 17·3. The effect of the bacteria is very marked in these cases in speeding up the growth of the Colpidia, although the final reproductive rate varies very little.

One of the great difficulties encountered in drawing deductions from the behaviour of organisms grown in artificial cultures is that the medium changes rapidly by the formation of waste products, whereas, under natural conditions, the medium would not accumulate these to any marked degree. It was thought that a culture might be freed to a considerable extent from the products of metabolism and, at the same time, be kept fairly constant by growing it inside a permeable membrane, surrounded by medium which could be frequently changed. For this purpose collodion sacks were made in the way suggested by Gates [1921]; that is, by dipping a gelatin capsule into collodion and later washing out the gelatin with hot water. If these are made carefully the collodion remains permeable even after autoclaving, though it shrinks to some extent during the process. Such sacks, containing 10 cc. of medium and surrounded by about 80 cc. of the same liquid, were inoculated with Colpidia in the ordinary way with most surprising results. Not only was the reproductive rate higher after the first day or two, but from the first count the ciliates in the membrane were very much larger than those in the control culture in a flask. Table III shows results from three typical cultures:

Table III. *Reproductive Rate to first maximum.*

Inoculum	Control	Membrane
40 per cc.	7·7	10·8
80 „	6·7	10·9
115 „	6·5	9·5

When the effect of the bacterial ratio was first noticed a count was made on a culture in a membrane and in a flask, when the number of bacteria proved to be very much higher in the membrane, even to the extent of forming a dense white deposit which made accurate counting impossible. The high results obtained from cultures made in these permeable membranes therefore seem to be due to the fact that bacterial growth is stimulated, probably by the provision of food substances contained in the sack. Such substances are derived from the gelatin capsules on which the sacks have hitherto been made, as the addition of collodion to a culture has no effect on its growth, while if a sack made up on gelatin in the ordinary way and then cut up is added to a flask culture the growth is once more increased.

Summary.

Experimental evidence is given that the number of divisions *Colpidium colpoda* undergoes in definite periods of time is intimately connected with the size of the bacterial population.

Further investigations on the relation between the size of the inoculum and the rate of reproduction demonstrate that the number of divisions steadily decreases as the number of animals inoculated increases.

REFERENCES.

Balbiani (1860). *Compt. Rend. Acad. Sci.* **50**, 1191.
Barber (1908). *J. Infect. Dis.* **5**, 379.
Churchman and Kahn (1921). *J. Exp. Med.* **33**, 583.
Cutler and Crump (1923). *Biochem. J.* **17**, 174, 878.
Gates (1921). *J. Exp. Med.* **33**, 25.
Maupas (1888). *Arch. Zool. Exp.* **6**, 165.
Peters (1921). *J. Physiol.* **55**, 1.
Robertson (1921). *Biochem. J.* **15**, 595, 612.
—— (1922). *J. Physiol.* **56**, 404.
Taliaferro (1923). *J. Exp. Zool.* **37**, 127.
Woodruff (1911). *J. Exp. Zool.* **10**, 557.