XXIII. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA.*

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Introduction.

That protozoa are important factors in soil economy is becoming increasingly evident since there is no question that large numbers of them pass through the various phases of their life history in the soil, and during their life influence the other members of the population. In a previous paper [Cutler, Crump and Sandon, 1922] it has been shown that the numbers of active protozoa of various species change rapidly from day to day without any obvious reference to gross environmental changes. Thus in the course of a year's daily counts in field soil, no correlation could be traced between the fluctuations in numbers of six species of protozoa and the rainfall, temperature or moisture content, and moreover the species appeared in the main to be living independently of one another.

It therefore appears that these variations in numbers are the expression of the animal's life cycle, or else that there is some obscure factor or combination of factors at work in the soil limiting activity. This second alternative is of course possible. If, however, the first suggestion is the true one growth in pure cultures should follow the same general course as obtains in the soil. For the two species tested, *Colpidium colpoda*, a ciliate, and *Oicomonas termo* (Ehren.), a flagellate, this is the case; for after the first few days of incubation, during which the culture is becoming populated, irregular variations in numbers occur, which are in every way comparable to the soil fluctuations.

The experiments detailed below were undertaken with a view to elucidating these changes, and to finding what are the factors in liquid culture determining the variations in reproductive rate and death.

From the point of view of soil biology such information is essential, but it is of added importance in respect of the various theories that have been advanced in explanation of growth. This has been dealt with extensively for the metazoa, metaphyta, and bacteria, but little is known regarding protozoa. Recently, however, Robertson [1921] has published experiments which he believes demonstrate that in certain ciliates growth is auto-catalytic

in character similar to that which has been recorded for higher organisms. Further research is necessary either to prove or disprove this view but the evidence adduced is not convincing.

The following account deals only with the earlier stages of growth in mass culture, later stages having been dealt with in detail by various authors.

In a future communication it is hoped to describe experiments on single cells isolated into drops of culture media of varying volumes.

METHODS.

The first essential in work of this kind is a rigorous standardisation of the cultural methods, so that any experiment can be repeated, or carried out in duplicate, with a reasonable expectation of obtaining consistent results, the organisms themselves being as far as possible the only variable factors.

Two organisms, whose source will be considered later, have been used throughout these experiments, *Oicomonas termo* and *Colpidium colpoda*. The method of estimating growth has been to count the number of animals in representative samples of the culture fluid in a counting chamber. The most convenient type for counting organisms of this size is the Cropper ruling, where an area of 2·5 sq. mm. is divided up into 625 squares of 0·04 sq. mm. area, the whole chamber being 0·1 mm. deep. The experimental error of the counts depends upon the number of animals counted, provided that the organisms are uniformly suspended in the fluid; then, if x is the number counted, the actual number present will lie between the limits $x \pm \sqrt{x}$ [Student, 1907]. In every case the number per cc. is given[1]. The animals are killed before counting by adding a small drop of lugol to the counting chamber. The experiments have all been carried out in the following way: 100 cc. flasks of quartz or Jena glass containing a known amount of medium (10 or 20 cc.) are used for the cultures. The number of organisms in the parent culture is counted and a quantity of liquid varying from 0·1 to 1·0 cc., according to the strength of inoculum required, is pipetted over into a new flask. In every case where the numbers of organisms in the sub-cultures have been counted immediately after inoculation the expected number has been found. The cultures are incubated at 19°.

Preliminary work suggested the following experimental conditions, variation in which would introduce errors:

1. Composition of medium.
2. Glass ware.
3. Aeration.
4. Temperature of incubation.
5. Strain of organism.
6. Food supply.

[1] The reproductive rate for any time is calculated from the formula: $\frac{\log B - \log A}{\log 2}$ where A = no. at beginning of time and B at the end.

1. *Composition of medium.* For comparative work organic infusions are wholly unsuitable, as it is impossible to reproduce them exactly. For this reason hay infusion has been discarded, although both *Oicomonas* and *Colpidium* thrive in it, and a synthetic medium has been used instead. In the first experiments on *Colpidium* Peters's "ammonium glycerophosphate medium" was used [Peters, 1921], or else one in which 0·06 % ammonium phosphate and 0·25 % saccharose were substituted for the glycerophosphate; the later experiments were carried out in a variation of Peters's glucose and lactate medium which contains the following compounds:

Na_2HPO_4	0·001 %	$MgSO_4, 7H_2O$ ...	0·0001 %
Ammonium lactate	0·01	$CaCl_2$	0·002
KCl	0·03	Glucose	0·04
NH_4Cl	0·03		

Colpidium has flourished on this medium for more than five months. According to Peters the optimum growth is obtained in this ciliate when the reaction of the medium is initially p_H 7·0 to 7·4. The medium has therefore always been adjusted to an initial reaction of approximately p_H 7·2, but satisfactory growth has been obtained at values outside these. Thus cultures starting at p_H 7·6 and 6·8 have shown apparently normal growth, and judging by the fact that Dale [1913] found the limiting values for growth in *Paramoecium caudatum* to be p_H 5·0 and 9·0 the range is probably very much wider.

Also a series of experiments made by S. M. Nasir in this laboratory showed that both ciliates and flagellates could live and reproduce in artificial media of p_H 3·9.

Peters's suggestion of adding phenol red in minute quantities to the cultures has also been followed, so that changes in acidity and alkalinity during the life of the culture can be noted; the routine procedure is to add two drops of the indicator to 20 cc. of medium. The final modification of the medium very gradually becomes alkaline as growth proceeds.

2. *Glass ware.* At an early stage in the experiments it was found that certain flasks, notably those made of Bohemian glass, had a bad effect on the growth of the organisms; in some cases no growth occurred at all. All the recorded experiments have been carried out in Jena glass or quartz flasks which give uniform results and do not affect the p_H value of the medium even after repeated sterilisation in the autoclave.

3. *Aeration.* Differences in aeration were found to have a marked effect on cultures of *Oicomonas*. Three series of cultures, each containing three parallels, were put up in the ordinary way: the first series were plugged with cotton wool, the second with corks through which a piece of bent glass tubing ran, and the third were attached to a filter pump so that a steady stream of air was drawn through them. In the second and third series the tubes leading to the outside air were so bent that no contamination of the flasks could occur. The results of this experiment are shown in Fig. 1. It is obvious that extreme aeration has a very disturbing effect on the growth of the cultures, and that

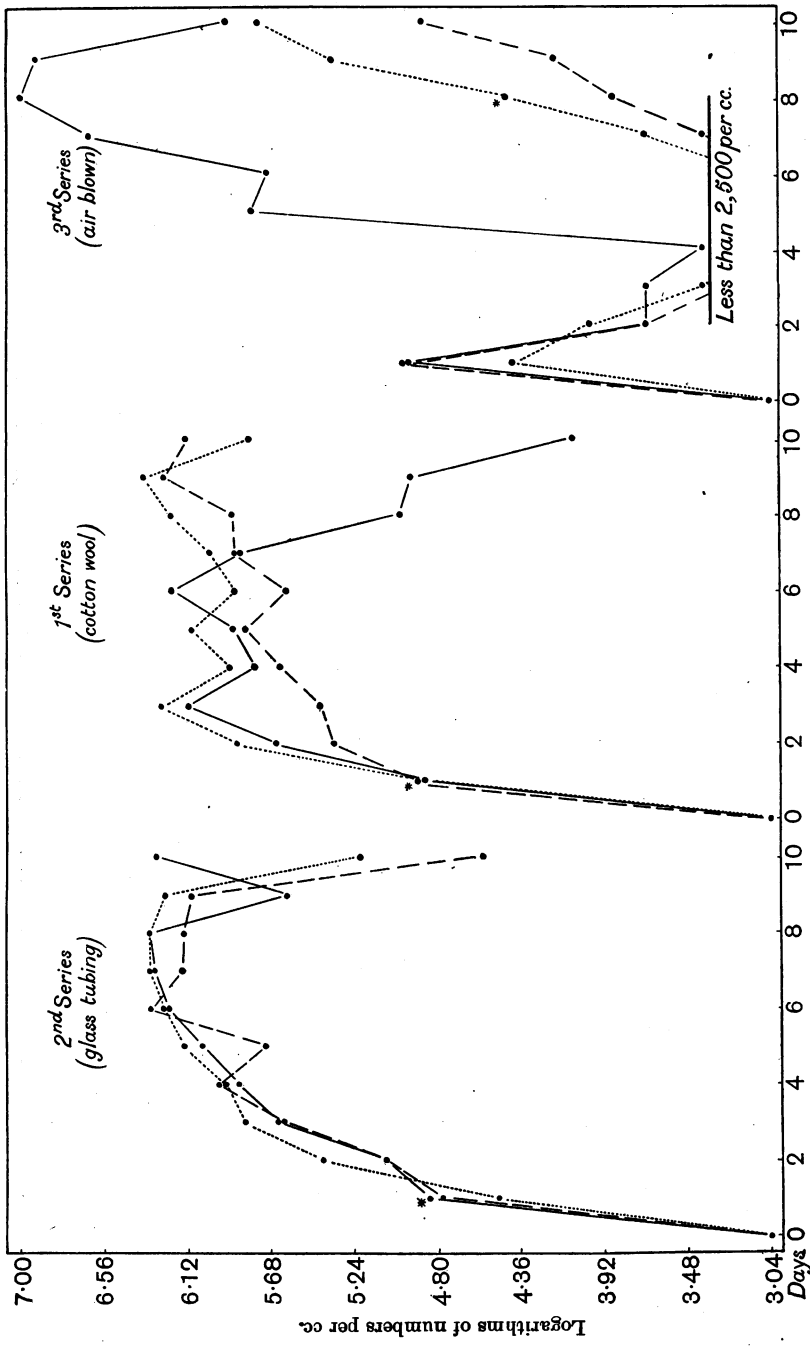

Fig. 1. Curves showing the effect of aeration on the reproduction of *Oicomonas*. The asterisks mark the points where conjugation forms were first observed.

better parallels are obtained when the aeration is absolutely constant (2nd series) than where there is even a slight variation (1st series).

4. *Temperature of incubation.* The incubation temperature throughout all the experiments has been as far as possible 19°; the effect of changing temperature is clearly shown from the results of the following experiments where two sets of flasks were incubated at 19° and 24° respectively (Fig. 2). Although

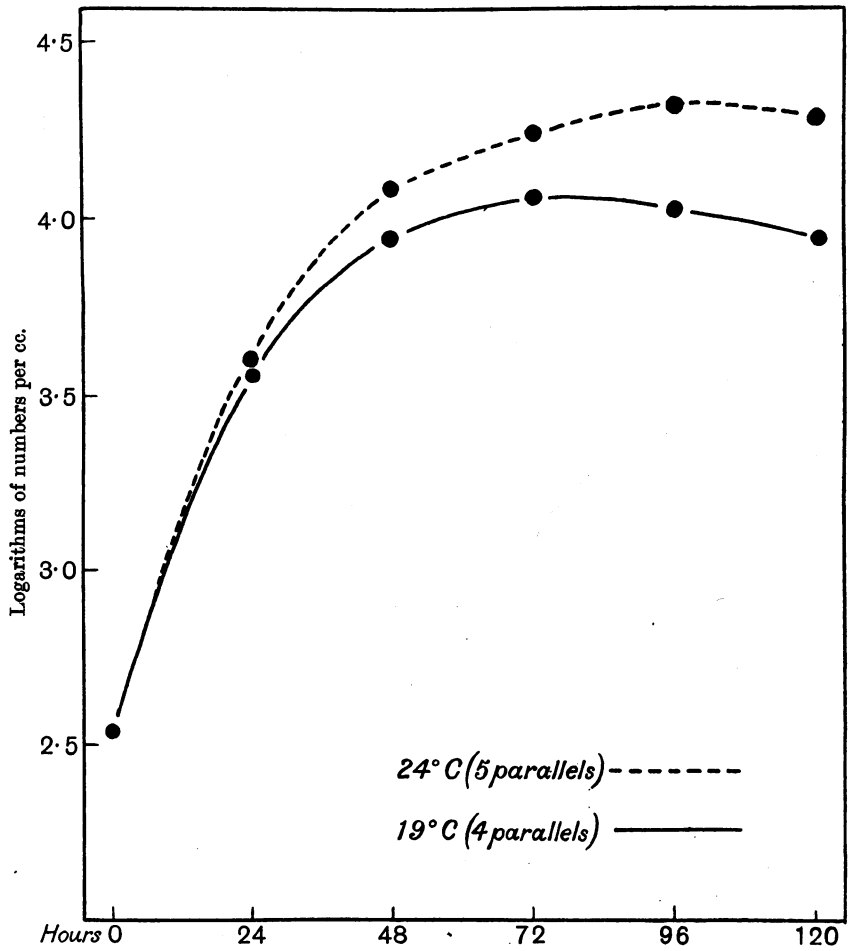

Fig. 2. Curves demonstrating the effect of temperature on the reproduction of *Colpidium.*

24° would appear to encourage more rapid, and even greater, reproduction in *Colpidium*, it is possible to maintain better experimental conditions at the lower temperature, as it approximates more nearly to that of the room and the shock of transferring the flasks from the incubator to the bench is very much less. In the experiment involving three hourly counts (cf. p. 180) a bath kept at 19° was arranged on the bench by the microscope and each flask was placed in it while the counts were made; McKendrick and Pai [1911] found this

a very necessary precaution where working with bacteria at incubator temperatures of 37°; at the lower temperatures it is probably an unnecessary refinement.

5. *Strain of organism.* The variation introduced by the strain of organism is probably considerable, since different strains show consistent differences in many characters. Thus, known differences in strain include such characters as size, rate of multiplication and frequency of conjugation [Jennings, 1920], resistance to heat [Hutchison, 1913; Jollos, 1913], the readiness with which conjugation occurs [Hopkins, 1922] and resistance to poisons [Jollos, 1913]. All the experiments on *Oicomonas* have been carried out on a single strain, the original culture being obtained from soil from Barnfield dunged plot; it is believed that the cultures came from a single cyst, though, owing to the small size of this organism, it is difficult to assert this positively. The cultures of *Oicomonas* are contaminated with at least three species of bacteria. Conjugation is of very frequent occurrence in *Oicomonas termo* thus making it from the point of view of the present work an unsatisfactory species to use. After the first appearance of the large conjugation forms (marked in each figure) the counts are subject to a considerable source of error; up to this point, however, they are accurate within the limits of experimental error. In the case of *Colpidium* all the experiments have been carried out on the same strain. The original stocks were given to us by Dr Peters and each had been derived in the first instance from a single cell. All our stocks of this ciliate are contaminated by a very small, stout, gram positive bacterium, which is present only in small numbers. Conjugation has never been seen in any of the cultures although they have been under close observation for more than six months, and only a very few isolated cysts have been found during that time.

6. *Food supply.* In the growth of a culture two factors are involved: the growth in size of the individuals, and the increase in their numbers, an increase, brought about in the cases under consideration, by binary fission. To attack the problem of growth is therefore by no means simple. The actual increase in size in any one animal depends in great part on the food supply[1], at least in young cultures; for this reason the most profitable method has seemed to be to provide a surplus of acceptable food and then to treat the increase of numbers as indicative of the actual increase in protoplasm. The *Colpidium* cultures have been fed either at inoculation or after 24 hours' growth with a pure culture of *Sarcina lutea* in sufficient quantity to ensure that a supply of it is present throughout the experiment. In the case of *Oicomonas* the contaminating bacteria provide abundant food. Even with these precautions the increase in the amount of living protoplasm in a culture can be found only by regarding both reproductive rate and average size of the animals. At present, however, our attention is confined to rate of reproduction.

[1] A culture of colpidia in which the mean length of 30 animals was 15μ and the mean breadth 5μ and in which there was a scarcity of bacteria was fed with sarcina; on the following day the mean dimensions for 30 animals were 20μ × 11μ.

Results.

The general course of a curve plotted from counts made at 12 or 24 hourly intervals on a mass culture of *Colpidium* is roughly sigmoid for a period varying from 2–6 days. There is then a fall in the numbers, and after this first maximum the curve becomes wholly irregular approximating closely to the type of curve obtained by counting protozoa in soil. As conjugation has not been taking place in any of the cultures it is obvious that death is the cause of the drops in the numbers. Experiment has shown, however, that death occurs in the earlier stages of the culture as well, and that the initial smoothly rising part of the curve can be resolved into an irregularly rising line by making counts at shorter intervals of time[1]. For this purpose three hourly counts were made extending over a period of 102 hours on eight cultures, four being made from a 24 hour old parent and four from one of 96 hours. In Figs. 3 and 4 curves derived from a typical culture from each series are shown. In each case the curve obtained from the 12 hourly observations shows a steady rise, while the 3 hourly line rises and falls irregularly. If only the 24 hourly observations are plotted, the curves are again completely changed, as in each case 2 maxima, which occurred at night, escape notice. It is not surprising that such being the case, it has not proved possible to apply the commonly accepted auto-catalytic formula[2] to any of the colpidium curves.

Death following inoculation.

A further complication, which must be recognised, before any general theory of growth and reproduction can be made, lies in the fact that death also occurs under certain conditions immediately after inoculation. Such is the case in cultures made from old parents. This is best illustrated by reference to the following experiments on *Colpidium* and *Oicomonas* (Figs. 5 and 6). In these, sub-cultures were made from the same stock culture at intervals of 12 or 24 hours and the numbers of protozoa in the sub-culture were counted after 12 and 24 hours and afterwards at 24 hourly intervals. During the first 12 hours after inoculation the sub-cultures from the older parents show a decrease in numbers; this, however, would pass unnoticed if the counts at 12 hours were omitted and the curve would then show the lag which is commonly found in bacterial cultures. Thus McKendrick and Pai [1911] with bacteria counted at half-hourly intervals found a lag when their cultures were derived from old parents (14 days as against 1–3 hours), which did not appear in cultures from young parents. Penfold [1914] found that the older the parent culture in the case of *B. coli* within limits, the longer is the lag period in the sub-cultures. In the protozoa Calkins [1919] has found in cultures of *Uroleptus* derived from single ex-conjugants, that old age in the parents leads to a series of low vitality, as evidenced by the duration of life and by reproductive

[1] In the case of certain bacteria Wilson [1922] finds that there is a normal death rate even during the period when the maximum rate of growth is proceeding.

[2] $\log \frac{x}{a-x} = k(t-t_1)$.

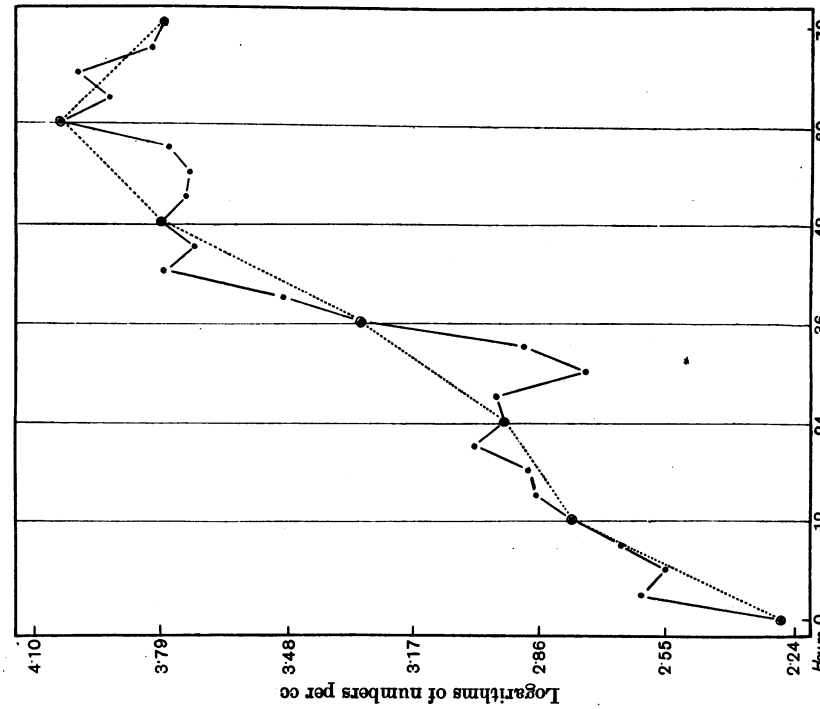

Fig. 4. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 24 hours.

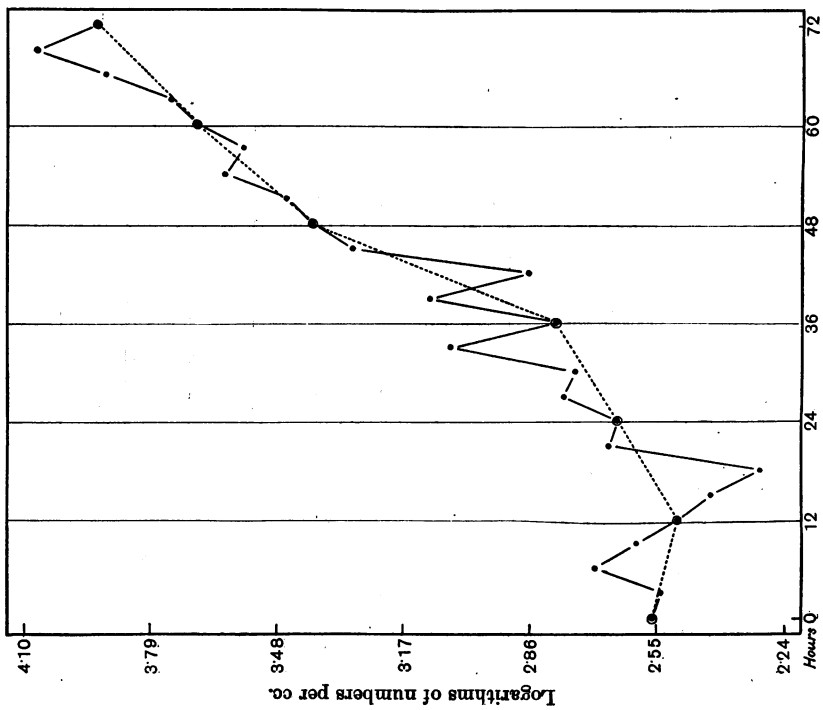

Fig. 3. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 96 hours.

ability, while young parents produce series of relatively high vitality. Robertson [1921] also adduces a certain amount of evidence bearing on this point. Starting from single individuals of *Enchelys* he finds that on an average

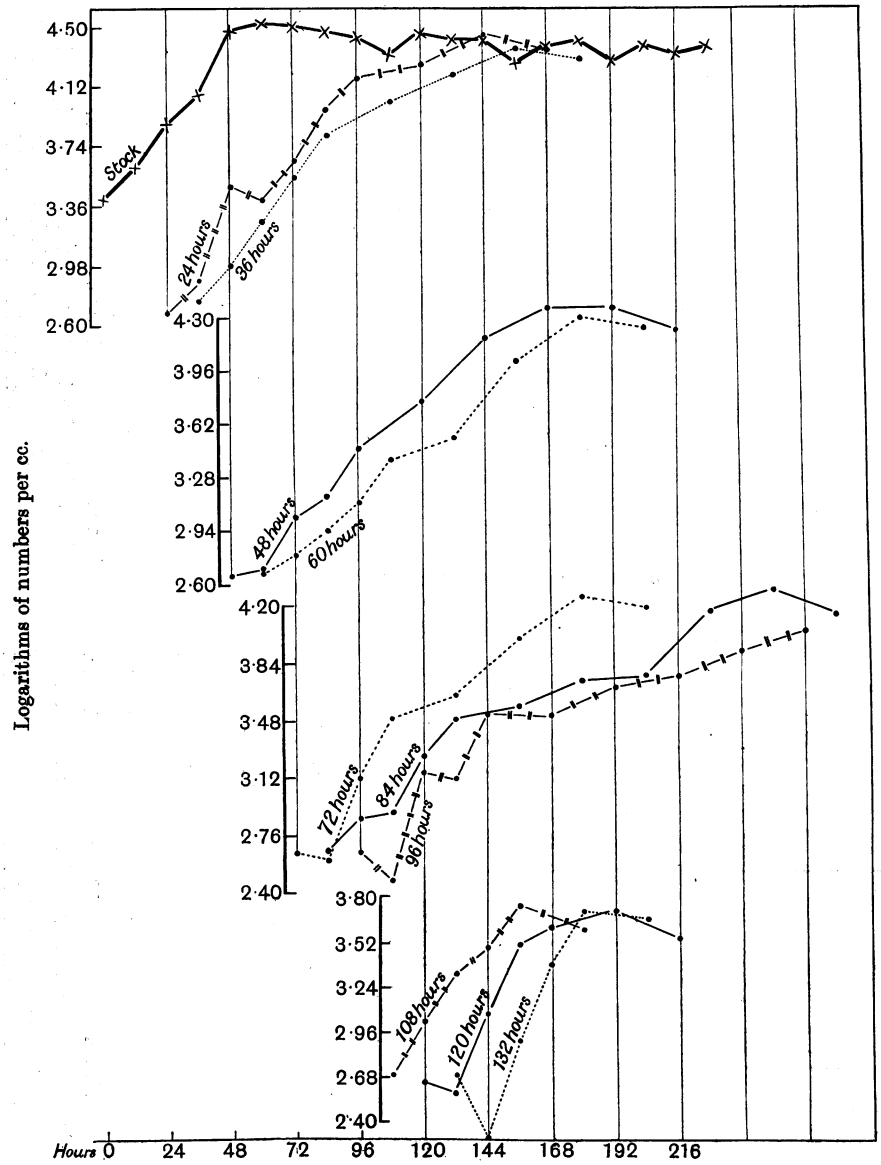

Fig. 5. Curves showing in *Colpidium* death following inoculation according to the age of the parent culture, which is written over each curve.

a one day old parent produces 38·4 in 24 hours, a two day parent 5·9, a three day parent 2·6 and a four day parent 2·0. In the case of the one day old parent, where he obtains the startling reproductive rate of 5·26 in 24 hours, he cites 27 cases in which the number of progeny produced vary from 11–105.

It is possible that in the case of the initial death the transfer of cells from a stale medium to a fresh one may involve too violent a change in the physical and chemical conditions, and that death is due to these causes. Certain facts, however, militate against such a suggestion. For instance, in the series of experiments with *Colpidium* (see Fig. 5) there is death on inoculation in both

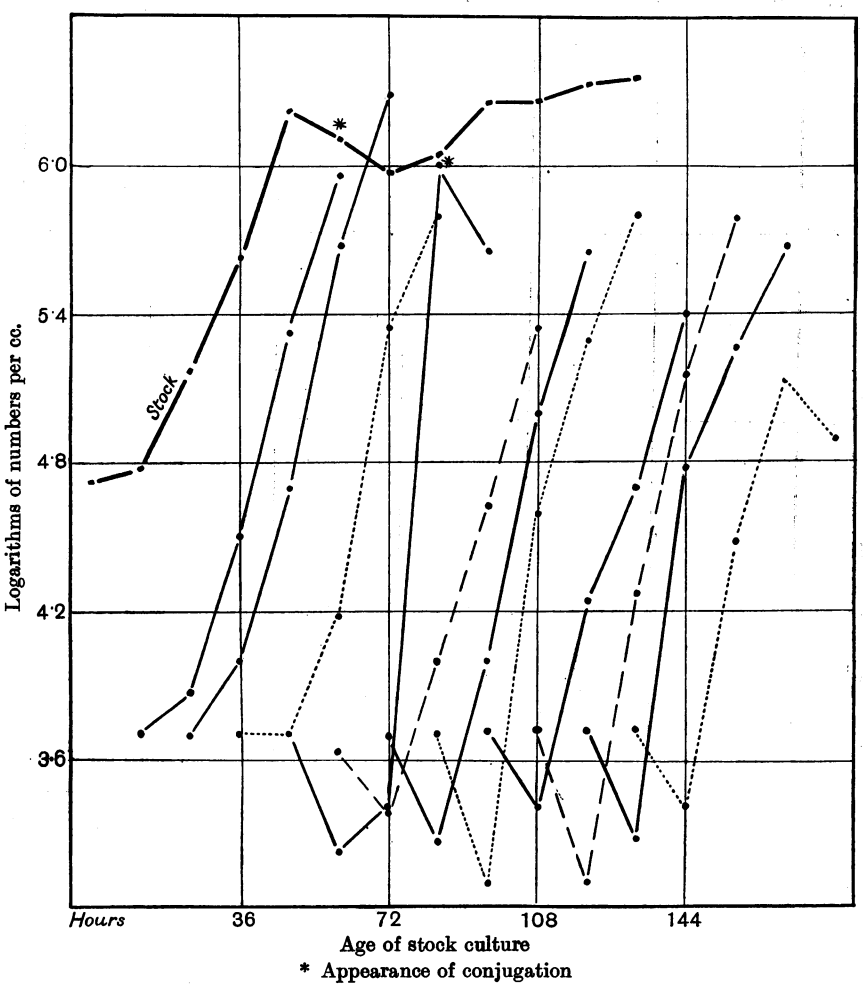

Fig. 6. Curve showing in *Oicomonas* death following inoculation according to the age of the parent culture. The asterisks mark the points where conjugation forms were first seen.

sub-cultures made from the 96 hour parent, but not in those made at 108 hours. It is interesting to notice that the stock culture decreased in numbers between 96 and 108 hours, but between 108 and 120 rose suddenly, which suggests that the initial rise in numbers in the 108 hour sub-culture was due to something inherent in the animals, which the change in medium is powerless to stop.

A further attempt has been made to clear up this point by sub-culturing

from the stock at various ages into its own fluid which is previously filtered. In every case there were very much lower numbers in the filtered liquid than in the fresh medium, but the results are vitiated by the fact that fresh medium when filtered gives lower numbers than control unfiltered medium, indicating that filtration changes the medium too much to permit of any deductions being drawn from such experiments.

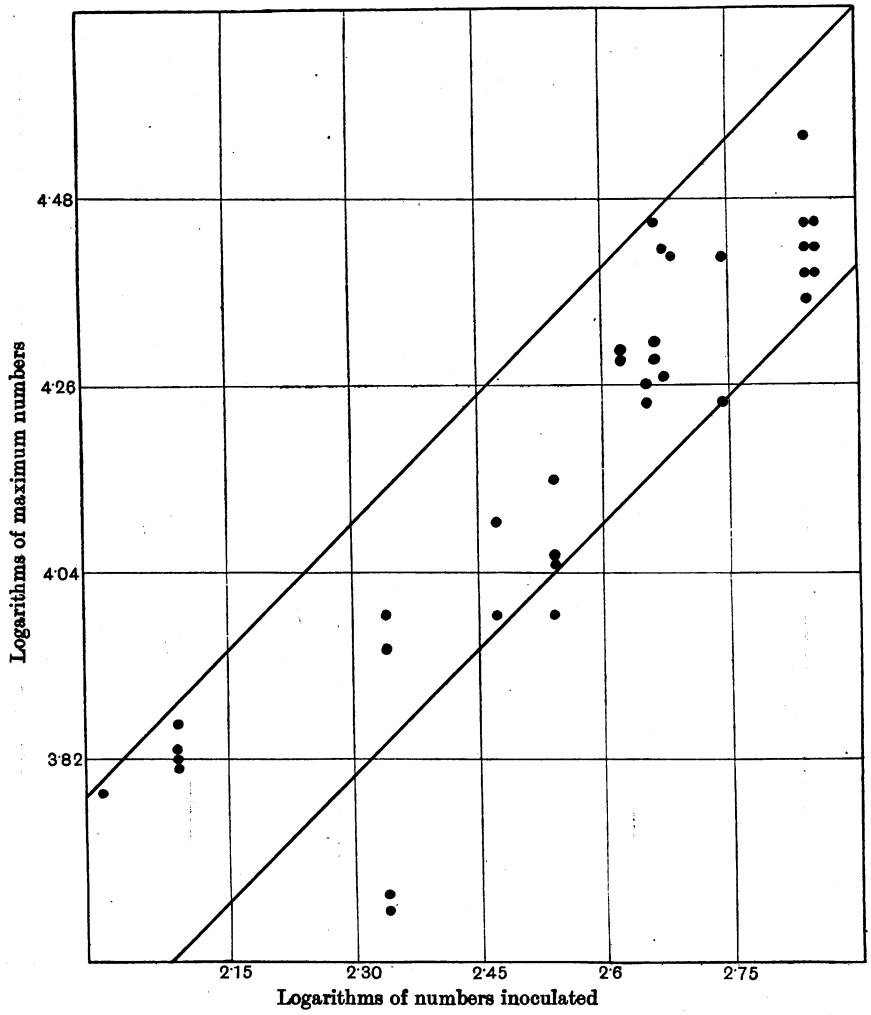

Fig. 7. Figure demonstrating the relation between the numbers of *Colpidium* inoculated and the maxima attained.

Constant reproductive rates.

Old age in the parent culture also influences the later growth of the sub-cultures (Fig. 5), the maximum numbers in such cultures tending to remain very much lower than they are in those from young parents. These lower

numbers which are obtained from old parents are undoubtedly in part due to the impaired vitality to which Calkins draws attention, but the initial death, by reducing the number from which the organisms have to rise, will also tend to keep the final numbers low. The number of organisms per cc. with which a culture starts, appears to influence it throughout its existence to a marked extent. In Fig. 7 the logs. of the numbers of animals inoculated are plotted against the logs. of the maximum numbers attained in the same culture. Each point therefore represents the average number of times that each animal has divided. In 91 % cases the points fall between the lines which include from 5 to 6 divisions.

The highest number ever reached by any of these cultures was about 35,000 animals per cc., but in other experiments there have been as many as 64,000 colpidia per cc.; therefore the comparatively low numbers reached in some cases cannot be in any way due to the environment itself, as is evident by the fact that young rapidly divided animals inoculated into such media are not killed as they would have been had the medium been toxic.

There is a great degree of variation in the size of the inoculum (100–700 per cc.), the age of the parent culture (24–48 hours) and the time taken in reaching the maximum number (48–168 hours) in the cultures represented. It is further of interest that the reproductive rate during the first 24 hours is very varied and apparently bears no relation to the final numbers attained.

Conclusions.

When the work now in progress on single cell cultures has been extended it is hoped to discuss in detail the experiments described above with especial reference to their bearing on the results already obtained from the investigation of protozoa living in the soil.

For the moment it is sufficient to point out how important it is that the experimental methods employed should be standardised if comparative results are to be obtained. To the physiologist this is self evident, but, judging by the papers already published, it has not been sufficiently appreciated by students of micro-biology. Also, to gain an insight into the life cycle of any species of protozoa, it is necessary to make observations, not only over a long period of time, but at frequent intervals. The three hourly count experiment recorded above shows how much, that is probably highly significant, is lost if the cultures are left unattended for even 24 hours. Finally it cannot be too strongly urged that in further work of this nature at least three, and preferably five, parallels to each experiment should be put up.

As regards the actual results obtained it would be premature to attempt an explanation of such phenomena as death following inoculation according to the age of the parent culture, and the constant reproductive rate of certain strains of *Colpidia*.

If in bacterial cultures a connection between the age of the parent and

death following inoculation also obtains it has an important bearing on the method sometimes used of differentiating between strains according to the time taken to ferment certain sugars.

In conclusion we wish to express our thanks to Dr Peters for providing us with the cultures of *Colpidium*, and to Mlle. Perey and Mr Sandon for valuable help during the short period counts.

Summary.

1. Methods are given by which it has been found possible to obtain comparable results when studying the reproductive rates of certain protozoa in mass cultures.

2. It is shown that within a relatively short period after inoculation, under certain conditions, a varying proportion of the organisms die; and that this is correlated with the age of the culture from which the inoculation was made.

3. By means of three hourly counts it was found that death occurs even during the period of maximum reproduction.

4. Evidence is supplied that in certain strains of *Colpidium* the rate of reproduction from inoculation to the maximum numbers attained is constant.

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