LXXIII. EXPERIMENTAL STUDIES ON CELLULAR MULTIPLICATION.

I. THE MULTIPLICATION OF ISOLATED INFUSORIA.

By THORBURN BRAILSFORD ROBERTSON.

*From the Department of Physiology and Biochemistry of the University
of Adelaide, South Australia.*

(Received August 11th, 1921.)

Statement of the Problem.

THOSE unicellular organisms which multiply by fission are, as Wassermann and others have pointed out, potentially immortal, since the protoplasm of the parent is contained in and shared between the daughter-cells. Furthermore, in the presence of unlimited food and environmental conditions which are otherwise suitable they are possessed of conceivably unlimited capacity for reproduction.

The actual conditions which attend the life of these organisms, however, never permit the full realisation of their capacity for multiplication, for even in the absence of any other check to unlimited reproduction, the ratio of population to food supply must sooner or later attain a point at which the further synthesis of protoplasm is an impossibility, and all obtainable food materials are requisite, or perhaps insufficient, to maintain the existing population.

Notwithstanding the imaginable potentialities of a Protozoon, therefore, the actual phenomena which are displayed by a culture in a limited amount of culture medium are not unlike those which characterise the life-cycle of the interdependent cell-masses which compose the Metazoa. In the one case, as in the other, the synthesis of protoplasm, starting from a single cell, becomes more and more rapid as the number of cells increases, until a maximal reproductive rate, or rate of growth, is attained. From this point onwards, for reasons which are not as yet perfectly understood, the cellular reproductive capacity progressively wanes until the population has attained a maximum.

It will subsequently be shown that a Protozoon culture exhibits youth, age, and the phenomenon of senescence, that is, not merely the slowing down of multiplication which is age, but that diminution of capacity to multiply, even under suitable conditions and in the presence of abundance of foodstuffs, which is senescence. The salient characteristics of the life-cycle of the higher animals are therefore also displayed by a culture of infusoria, and although the individual Protozoon is potentially immortal, the society comprised in a

given culture is subject to the law of mortality which sets a term to the existence of more complex cell societies.

Those factors which are fundamental to and reside in the nature of protoplasm itself must operate alike in a Protozoon culture and in the specialised cell-aggregates which form the Metazoa. A study has therefore been undertaken of the various factors which determine reproductive rate in cultures of infusoria, with a view to the application of information so obtained to the interpretation of the phenomena of growth in higher animals.

There are especial advantages in approaching the problems of growth from this angle, because the units which comprise a culture of infusoria are separable from one another without the slightest disturbance of the normality of their environment. The several units are alike, and their reproductive capacities may be directly ascertained by counting their descendants in a given period of time. Finally the entire cycle of development, from the single cell to the multitude of its descendants which are no longer able to increase, occupies a period of but a few days.

Methods of Investigation.

The species of infusorian employed in these experiments was *Enchelys farcimen* Ehr. It is an ovate or pyriform organism, finely ciliated throughout, the cilia being of uniform length over the greater part of the body, but somewhat longer in the oral region. The mouth is at the narrowed anterior extremity, directed somewhat to one side. In old and "starved" cultures the organism is usually about 50μ in length and one-third as broad, but in well-fed cultures which are only 24 hours old, or even 48 hours old when for any reason development has been delayed, the organisms are much broader and of a pear-shaped or even cubical outline. The pear-shaped organisms measure from 50μ to 70μ in length and from 35μ to 50μ in breadth. Recently divided organisms are somewhat more than half the length of the fully grown individuals and globular or pear-shaped. Exactly similar changes of outline with abundance or scarcity of food have been noted by Jennings in cultures of *Paramoecium* [Jennings, 1910].

All of the organisms employed in these experiments arose, by repeated propagation of single individuals into fresh culture media, from two "wild" individuals originally isolated from a culture of chrysanthemums. The descendants from these two individuals have been separately propagated for over 200 generations, forming an "A" and a "B" series of cultures. Throughout this period no uniform difference whatever has been observed in the rates of multiplication of the two strains, the few instances in which individuals simultaneously isolated from "A" and "B" cultures yielded decidedly differing numbers of descendants in 24 hours being in every case traceable to differences in the histories of the parent cultures or in the treatment of the subcultures.

The culture medium employed was a hay infusion formed by heating five grams of chaffed hay in 100 cc. of tap water to boiling and then continuing

the heating on a boiling water-bath for 30 minutes. The infusion was filtered, 0.4 cc. of $N/10$ Na_2CO_3 was added to every 10 cc. of the filtrate, and the mixture usually employed as soon as it was cool, being made up afresh for each day's subcultures. This is subsequently referred to as "Normal Hay Infusion." In some experiments this fluid was allowed to stand at room temperature in a shallow covered layer, for from 24 to 72 hours, at the end of which period it is thickly populated by bacteria which have arisen from the spores which are not destroyed by boiling. This fluid was filtered through paper to free it from the zoogloal masses floating upon its surface, the filtrate constituting what is subsequently referred to as "Bacterised Hay Infusion."

Subcultures were prepared by removing from the parent culture a minute drop, averaging about 0.002 cc. by means of a pipette drawn out to a fairly wide capillary bore. This drop, usually containing from ten to thirty individuals, was placed in the centre of a depression in a microscope slide and diluted by the addition of a larger drop, averaging about 0.04 cc. of the fresh culture medium. Another minute drop was abstracted from this mixture and the process repeated until a single individual was obtained. Since it is possible to overlook an individual swimming close to the curved edge of a drop, and abnormal results in some of the earlier experiments were traced to this origin, the practice was adopted of invariably *re-isolating* the individual by diluting the drop in the manner indicated and abstracting small samples until one was found again to contain the single individual. This drop, measuring about 0.002 cc. was then diluted by the addition of two large drops or 0.08 cc. of culture fluid, forming the final subculture. It will be observed that even if the first small drop isolated from the parent culture chanced to contain but a single individual, the final dilution of the parent culture fluid in the new subculture could not be less than 1 in 800 and, of course, it was usually much greater.

The slide containing the culture was placed upon a pad of filter paper, soaked in water, in a petri dish, the edges of the dish being vaselined to prevent evaporation.

The food of infusoria consists, not of the soluble substances which are present in the infusion, but of the bacteria which subsequently develop therein. Hence the factor which determines the food supply is the degree of bacterial infection in the culture fluid. For reasons which will be evident in the sequel, a culture heavily infected with bacteria may cause very rapid multiplication of infusoria. Herein lies one of the main difficulties in standardising the culture medium employed. For if the culture be too nearly sterile infusorial development is very slow, and if it be allowed to accumulate a large bacterial population before being employed the relative abundance of bacteria in different infusions may conceivably vary very greatly from time to time. It was felt that the nearest practicable approach to standardised conditions was afforded by employing for each day's subcultures freshly-prepared "Normal Hay Infusion," containing, presumably, comparable numbers of bacterial

spores. In the course of the experiments it was found necessary, however, to sterilise the glassware employed to contain the infusion, the pipettes employed for isolation and dilution and the slides upon which the cultures were grown. Otherwise a film of bacteria on the surface of the glass may occasionally lead to irregular results. The technique employed was essentially similar to that described by Jennings [1913, 2]. Containers, measuring cylinders, etc., were rinsed with ether which was subsequently washed out with repeated jets of distilled water.

No attempt was made, in the majority of the experiments, to maintain a constant temperature, because the thermal death-point for these infusoria is so low that for the greater part of the year in South Australia room temperatures represent approximately optimal conditions.

The organisms were isolated and counted under a magnification of 64 diameters, employing a moderately bright, artificially illuminated field, against which they appeared relatively opaque.

The Influence of the Age of the Parent Culture upon the Multiplication-rate in Subcultures.

The rate of multiplication of individuals isolated from cultures over 48 hours old is very much slower than the rate of multiplication of individuals which are isolated from younger cultures. This fact is displayed in Table I and in the curve (Fig. 1) which summarise the various results obtained during the period of eight months throughout which these cultures have been the subject of experimentation. The temperatures were not uniform and for this reason there appears little utility in calculating the probable errors of the averages obtained, since the observed variation of the results depended, not merely upon the inherent variability of the reproductive power of the cells themselves, but also, in part, upon varying extraneous factors. Nevertheless the results are sufficiently uniform to display the essential phenomena, and the conditions of propagation were alike for each age of parent culture, so that the averages obtained in each class are comparable with one another.

The initial rapidity with which the reproductive capacity of isolated individuals falls off with the age of the parent culture is very remarkable. This rapid loss of reproductive power during the first 48 hours is succeeded by a slow but definite further loss during the following 48 hours. The slight recovery indicated in the last column of Table I (parent cultures 5 to 9 days old) may be merely apparent and due to the variability of the individual results and an insufficient number of observations to establish a true average. I am inclined to believe, however, that this increase is not apparent but real, and that the starvation to which the organisms in these very old cultures have been subjected renders them exceptionally sensitive to certain chemical conditions which, as we shall see, favour rapid multiplication. Experimental data bearing upon this point are not as yet sufficiently numerous, however, to enable me to affirm this view with certainty.

Table I. *Cultures in Normal Hay Infusion.*

Number of individuals produced in 24 hours from a single individual isolated from parent cultures of the following ages:

1 day old		2 days old		3 days old		4 days old		5 to 9 days old	
16	14	4	8	8	2	2		2	
16	11	1	12	7	2	2		2	
33	71	4	8	2	2	2		4	
32	46	28	8	2	8	2		2	
64	31	10	24	1	4	2		2	
93	18	8	2	1	2	3		1	
105	14	9	4	1	1	1		4	
96	14	8	3	1	7	1		2	
40	18	2	2	4	2	4		1	
52	21	2	8	4	2	4		2	
30	56	2	4	4	2	2		2	
24	24	3	4	2	2	2		4	
56	16	2	4	2	2	1		2	
26		8	1	2	4	2		1	
		6	1	1	4	1		8	
		4	3	1	4	1		6	
		4	1	1	2				
		4		1	3				
				1	1				
				1					
Averages:	38.4	5.9		2.6		2.0		2.8

Fig. 1. Showing the decrease of the multiplication-rate of isolated infusoria with increasing age of the parent culture.

The loss of reproductive capacity in individuals isolated from old parent cultures is due to changes which have occurred in the organisms themselves, and not to any retardative substances associated with the organisms in the parent culture and introduced together with the isolated individuals into the subculture. This is shown by the fact that repeated washing of the isolated individual in freshly-prepared hay infusion or in tap water or in faintly alkaline distilled water (2 cc. of $N/10$ Na_2CO_3 to every 100 cc. of water) does not restore its reproductive capacity. Moreover the parent culture fluid is abundantly capable of supporting and permitting reproduction of infusoria if those already inhabiting it are removed.

It is not easy to remove these infusoria from a culture by mere filtration through paper, because *Enchelys* is able to pass through the pores of even hardened filter paper. It was not desired to remove them by filtration through a Berkefeld filter, because this procedure would also remove the bacteria which form the food of the infusoria. But by heating to 50°C . for a few moments the infusoria are all killed and, having been immobilised, they are readily removed from the fluid by filtration through paper. The bacteria, at least the majority of them, are not killed by exposure to this temperature, and they pass freely through rapid-filtering paper. Now on subculturing single individuals into this fluid normal reproductive rates are observed, comparable to those observed in simultaneously prepared subcultures into bacterised hay infusion which has never been tenanted by infusoria. The following experiment illustrates this fact:

Bacterised hay infusion which had stood for two days at room temperatures was heated to 50°C . and then immediately cooled and filtered. Infusorial "mass cultures," two and five days old respectively, and densely inhabited by infusoria, were similarly heated to 50°C . and then filtered. Single individuals from the same parent cultures (A and B series), 48 hours old, were isolated into each of these culture media:

Culture number	Culture medium	Number of individuals after 24 hours
197 A	Bacterised hay infusion	8
197 B	" "	16
200 A	Filtrate from 2-day infusorial culture	8
200 B	" "	11
201 A	Filtrate from 5-day infusorial culture	8

The origin of the loss of reproductive capacity in old cultures is therefore to be sought in the density of the population, and not in any failure of the culture fluid to supply necessary nutrients or in any accumulation of products adverse to the multiplication of infusoria. The most probable explanation of this fact would appear to be that the nutritive requirements for the maintenance of the numerous infusorial population in an old culture have overtaken the rate of the reproduction of bacteria, so that the bacterial population is consumed just as rapidly as it is produced and the infusoria are thereby maintained at a nutritive level but little above the level of starvation. When the infusoria are removed the bacteria are permitted an opportunity to multiply before the new infusorial population attains a sufficient density to check them. We may

infer, therefore, that a period of existence at a low nutritive level is the factor which is actually responsible for the loss of the reproductive capacity of infusoria in old cultures.

This interpretation of the phenomenon is supported by the direct observation that the bacterial population of old infusorial cultures is very small and speedily increases after removal of the infusoria, and it is furthermore supported by the frequently-repeated observation that it is the density of the infusorial population rather than the age of the culture in days which determines the reproductive capacity of its members. If for any reason such as lowered temperature, or a low reproductive capacity of the parent culture, development of infusoria in a subculture is retarded, then at two days the individuals isolated from it may display the reproductive capacity normal to individuals isolated from a 24-hour culture. In this way, subculturing alternately from old and young cultures, a rhythm of reproductive energy is readily induced and subcultures alternately exhibit populations of two and of from 16 to 32 individuals respectively in 24 hours after isolation. It is mainly to this fact that the wide variations of reproductive capacity are due which are displayed by individuals isolated from different parent cultures of equal age (cf. Table I).

The Influence of Preliminary Bacterisation of the Culture Medium upon the Multiplication-rate of isolated Individuals.

Bacterisation of the hay infusions by preliminary standing at room temperatures for from 24 to 72 hours remarkably stimulates the reproductive capacity of the isolated organisms, and it delays, but does not completely prevent, the loss of reproductive capacity which occurs with age of the parent culture (Table II and Fig. 1). Even in the presence of superabundance of food, therefore, the individuals isolated from old parent cultures suffer a definite loss of reproductive capacity which appears to be analogous to the senescence

Table II. *Cultures in Bacterised Hay Infusion.*

Number of individuals produced in 24 hours from a single individual isolated from parent cultures of the following ages:

2 days old		3 days old
34	16	8
29	8	8
28	31	1
34	60	2
35	8	3
17	8	2
26	7	8
17	4	3
8	11	16
11	16	9
8		
Averages: 19.9		6.0

which overtakes the tissue-cells of the higher Metazoa with advancing age. Here again, however, very old (*i.e.* starved) parent cultures may show a surprising recovery of reproductive capacity in bacterised infusions. Thus in one experiment individuals from cultures of the "A" and "B" strains which were no less than 8 days old had produced 33 and 31 individuals respectively 24 hours after isolation into a bacterised infusion.

It will be seen, on comparing the results enumerated in Tables I and II, that the reproductive capacities of individuals isolated into bacterised cultures are even more variable than the reproductive capacities of individuals isolated into normal hay infusion, initially sterile except for bacterial spores and the small contamination introduced together with the isolated infusorian. The same fact has previously been noticed by Jennings and it appears probable that the extent to which previous bacterisation of the infusion favours the multiplication of infusoria depends upon the ratio of bacterial to infusorial population and upon the period and temperature of the previous incubation. Experiments designed to ascertain the precise method of preliminary bacterisation which produces maximal acceleration of multiplication are at present in progress.

Heating the bacterised infusion to boiling-point, or even for 90 minutes on a boiling water-bath just before its employment, does not diminish the multiplication-rate of individuals isolated into it. The following is illustrative of many experiments:

Culture numbers 150 and 151. The parent culture was two days old. The hay infusion had stood for two days at room temperature:

Culture number	Number in subculture after 24 hours		Culture fluid
	A	B	
150	35	17	Bacterised hay infusion
151	26	17	Same, boiled before using

Evidently, therefore, living bacteria are not essential for the occurrence of the accelerated multiplication. Doubtless killed bacteria are not less available than living ones as sources of food materials, but although the final number of infusoria produced in a given culture is sooner or later determined by the number of bacteria, dead or living, which are present, yet the initial accelerative action of bacterised culture media remains very pronounced even after passage through a Berkefeld filter, which deprives it of bacterial content. This is illustrated by the following, typical of a number of experiments:

Cultures 234, 236 and 237. The parent culture was two days old. Cultures 234 (A and B) were isolated into normal hay infusion, 236 (A and B) into hay infusion which had been bacterised for two days and then filtered through a Berkefeld filter, 237 (A and B) into the same bacterised infusion, without filtration. The cultures were examined at frequent intervals and at the end of six hours division had occurred in each of the cultures 236 (A and B) while there was as yet no sign of division in the cultures 234 (A and B) or 237 (A and B). In 22 hours the numbers in cultures 234 and 236 were equal (4 in each) while the cultures 237 (A and B) were increasing more rapidly (8 and 5 respectively). In 28 hours the individuals in the unbacterised infusion numbered 8 and 14, in the filtered bacterised infusion 4 and 4, and in the unfiltered infusion 20

and 16. The acceleration due to bacterisation was not, in this instance, very pronounced, but it occurred first of all in the bacteria-free medium and only ceased to manifest itself when actual deprivation of nutriment compulsorily brought multiplication to a standstill. At 22 hours, when the numbers in the various cultures were approximately equal, the organisms inhabiting the filtered fluid were of the thin, actively-swimming, "starved" type, while those inhabiting cultures 234 and 237 were of the "fat" type and very sluggish in movement.

The interest of the above experiment lies especially in the observation of the moment at which the first division occurred, and the fact that it occurred earliest in the filtered bacterised medium. The following illustrates more strikingly the accelerative effect of soluble bacterial products, without confusion of the comparison by the secondary effects of unequal food supply:

Cultures 259 and 263. The parent culture was three days old. The hay infusion was bacterised by standing for 48 hours at 23° C. and then filtered through a Berkefeld filter. The filtrate was mixed with an equal volume of normal hay infusion:

Culture number	Number of individuals in subcultures in 24 hours		Culture fluid
	A	B	
259	1	1	Normal hay infusion
263	11	16	Same plus an equal volume of bacterised and filtered infusion

The accelerative effect of the bacterised infusion is not therefore dependent upon the presence of bacteria, whether living or dead, but upon the presence of soluble products arising from the inhabitation of the medium by bacteria.

It might be imagined that the change in the medium induced by an abundant population of bacteria consists in nothing more than an adjustment of the reaction to a more favourable hydrogen ion concentration. The quantity of Na_2CO_3 added to the normal hay infusion was, however, that which was found by repeated trials to yield the optimal reaction for multiplication. Saturation of the normal hay infusion with carbon dioxide increases the reproductive rate a little, but not to any extent comparable with the acceleration which may be produced by bacterisation. Moreover the accelerative agent is not volatile; it is not discharged from the bacterised fluid by boiling, nor can it be conducted by distillation into normal hay infusion.

Since the accelerative effect is independent of the supply of foodstuffs and may actually be evidenced by relatively starving organisms, the action of this substance presents analogies to the accessory rather than to the primary or calorific foodstuffs. The recent investigations of Williams [1919], Bachmann [1919], and of Funk and Dubin [1920] have shown that aqueous or acid alcoholic extracts of a variety of vegetable tissues, such as autolysed yeast or lucerne, contain a substance which has a marked accelerative effect upon the multiplication of yeast cells, and because of a rough parallelism which exists between the content of water-soluble accessory in such extracts and their stimulating effect upon the growth of yeast, it has been proposed to employ the increase in the rate of multiplication of yeast as a measure of the vitamin content of extracts. More extensive comparisons by Eddy, Heft, Stevenson and Johnson [1921], have shown, however, that the yeast tests

merely approximate the results of feeding tests on rats, the agreement being exact only within the range of dilutions between which the accelerative effect increases rapidly with concentration. Above this range the higher concentrations of the extract do not produce a corresponding increase in the reproductive rate of yeast, and an optimum concentration exists beyond which the curve of stimulation falls. No such phenomena are exhibited in feeding experiments with rats, so that the substance which affects the reproductive rate of yeast would not appear to be identical with the water-soluble vitamin. Furthermore Emmett and Stockholm [1920] have recently shown that the yeast-stimulating substance is thermostable, resisting boiling temperature for 90 minutes, while the water-soluble vitamin is destroyed under like conditions.

The substance in bacterised infusions which accelerates the reproductive rate of infusoria is similarly thermostable and may possibly be identical with the substance in autolysed yeast extracts, etc., which accelerates the reproductive rate of yeast. In its thermostability this substance (if, indeed, it is identical with any of the hitherto identified classes of food accessories), resembles the fat-soluble accessory rather than the water-soluble accessory, but until we possess a more exact knowledge of the relationship of the stimulative substance in bacterised hay infusions to the known food accessories on the one hand, and the yeast-stimulating substance on the other, I prefer for brevity of reference to employ the designation "X-substance."

Incidentally these results throw light upon the origin of an old misunderstanding. The original experiments of Engelmann [1876] and Maupas [1888, 1889] were held to have proved that asexual reproduction in infusoria could not continue indefinitely, because slide-cultures propagated repeatedly without conjugation died out, owing to total loss of reproductive capacity, in from 200 to 300 generations. On the other hand it was in some instances found that conjugation with a "wild" individual caused a remarkable access of vigour and reproductive capacity. Hence it was inferred that conjugation exerted a rejuvenating and invigorating effect upon the stock. Jennings [1913, 1], however, has demonstrated that if otherwise similar cultures be compared, those in which conjugation has occurred have actually an average reproductive capacity far *lower* than that of individuals which have never undergone conjugation. The offspring arising in conjugating cultures are, however, much more variable than those which arise by asexual reproduction, so that an occasional individual may chance to display a high reproductive rate. So far from exerting a rejuvenating effect upon the stock, conjugation brings about many abnormalities and greatly increases the death-rate among isolated individuals.

The loss of vigour in asexually propagated slide-cultures does not arise, therefore, from the absence of conjugation. According to Woodruff [1911] it is due to monotony of the environment, and he has succeeded, owing, he believes, to the fact that he repeatedly varied the culture medium, in propagating a culture of *Paramoecium* asexually, without any loss of reproductive

capacity, for over two thousand generations. In reading his description of the culture media employed, it appears very possible that the variation in the medium which, from time to time, resulted in re-invigoration of his cultures, may have been a favourable degree of preliminary bacterisation. He describes his culture media in the following words: "From May 1907, through January 1908 the culture medium consisted of infusions of hay and grass, but no particular care was taken to keep the medium the same from day to day.... In some cases the liquid was used as soon after it had been boiled as it had attained the room temperature, in other cases it was allowed to stand in a stoppered flask for twenty-four hours or more. But from February 1908 to the present time a more varied culture medium has been used... materials for the infusions were taken practically at random, from laboratory aquaria, hay infusions, ponds, etc. The infusions were thoroughly boiled to prevent the contamination of the pure lines of the pedigree culture by wild individuals."

Since boiling does not diminish the stimulating effect of bacterised cultures and many of the infusions described by Woodruff must have been heavily infected with bacteria before boiling, intermittent stimulation of reproductive capacity through the presence of "X-substance" in the culture media must evidently have occurred.

For over 200 generations my laboratory cultures of *Enchelys*, propagated asexually, have shown no consistent diminution of reproductive capacity. It is, however, very easy to cause a rapid loss of reproductive capacity by repeatedly subculturing from old and "starved" cultures into sterile hay infusions. In two or three generations a high mortality prevails among isolated individuals, and the reproductive rate of those which survive is very low. Immediate restoration of viability and reproductive rate is achieved by cultivation into bacterised infusions, or, more slowly, by repeated propagation from young cultures. It is of very great interest in view of results which are about to be described, that whereas isolated individuals from cultures which have repeatedly been propagated from old parent cultures almost invariably die, "mass-cultures" propagated by introducing 20 or 30 individuals simultaneously into the infusion invariably survive. Either some of the individuals retain sufficient viability to populate the culture, or the contiguity of numerous cells promotes the viability and multiplication-rate of each of them. In view of what follows the latter must be regarded as a probable explanation.

It has been observed by Jennings, who employs freshly-extracted and boiled hay infusions and carefully sterilises all of the glassware employed, that "there are some stocks (of *Paramoecium*) which will not stand long continued cultivation on slides at all, although they live perfectly in mass-culture." In my experience it makes no difference whether the culture inhabits a drop of infusion contained in a depression in a slide, or the same fluid contained in some larger vessel, provided only that a number of individuals are simultaneously introduced into the new culture liquid.

The Time-relations of Multiplication in Cultures of isolated Infusoria.

It has been shown in a number of investigations [Robertson, 1908, 1913, 1915, 1916; Ostwald, 1908; McKendrick, 1910] that the time-relations of growth, whether in bacterial cultures or in the tissues of the higher animals, are similar to those which obtain in chemical reactions which produce their own catalysors. The bacterial count in cultures of bacteria, or the total weight of the body in higher animals or plants, increases at first very slowly, but as the cells increase in number the velocity of multiplication increases proportionately until it attains a maximum, from which it falls off to the condition of stasis which marks the end of multiplication in the culture or of growth in the animal. In the *Vertebrata* periods or "cycles" of growth alternate with periods of relative stasis, but each of the growth-cycles, such as for instance that which initiates *in utero* and culminates towards the end of the first year of extra-uterine life in man [Robertson, 1916], reproduces with truly surprising exactitude the quantitative characteristics of an autocatalysed chemical reaction, the weight of the animal or infant taking the place of the mass or concentration of the product in the chemical reaction-formula.

Precisely similar quantitative relationships are exhibited during the growth of a culture of infusoria. There is a more or less prolonged "lag-period" during which multiplication is very slow or apparently arrested. This is succeeded by a progressively increasing rapidity of multiplication which, after passing through a maximum, subsequently sinks and ultimately falls to zero level. Hence multiplication is always much less rapid during the first 24 hours after isolation than it is during the second 24 hours, even in bacterised infusions. The following are illustrative results among a very large number recorded during the progress of these experiments:

Normal Hay Infusions.

Culture number	Individuals in subculture after		Number of divisions during	
	24 hours	48 hours	24 hours	48 hours
51 A	2	30	1	5
51 B	1	10	0	3
215 A	1	26	0	4.5
217 A	2	61	1	6

The same relationships are exhibited when shorter time-intervals are employed in more rapidly multiplying cultures. The curves in Fig. 2 illustrate some such instances, and it will be seen that they correspond closely to the earlier or ascending part of the curve which displays the relationship between the mass of the product and the time in an autocatalysed chemical reaction. The positions of points on the further continuation of the curve are not easy to determine on account of the difficulty of counting so many actively moving individuals, and I have as yet made no attempts in this direction. The data set forth above, however, taken in conjunction with the readily ascertainable fact that reproduction ceases altogether after the population of the culture

has become very numerous, show that the reproduction of infusoria displays the general autocatalytic characteristics which are encountered in other types of growth.

In the case of infusorial cultures, however, a special complication is introduced by the fact that the food of the organisms is supplied by bacteria which are simultaneously multiplying in the culture. The possibility is thus suggested that the autocatalytic character of the multiplication of infusoria may merely be the reflection of the autocatalytic relations governing the multiplication of bacteria, which have been evaluated by McKendrick [1910]. Part of the effect must doubtless be attributable to this source, but that it is not entirely attributable to the autocatalysed multiplication of food-materials is shown by the fact that the auto-acceleration is displayed to a pronounced degree in bacterised infusions. The "lag-period" is somewhat less in duration, but still very perceptible, and the reproductive rate, although more rapid in fresh hay infusions, undergoes progressive acceleration with the lapse of time and increase of the population. The following is an illustrative experiment, which is also depicted in curves 237 A and 237 B in Fig. 2.

Fig. 2. Showing the auto-acceleration of multiplication-rate in cultures arising from single isolated infusoria.

Bacterised Hay Infusion, unfiltered.

Culture number	Number of divisions which had occurred after			
	6 hours	22 hours	24 hours	28 hours
237 A	0	3	3	4.5
237 B	0	2	3	4.0

Thus in the first six hours after isolation, and in the presence of abundance of food, no divisions occurred, but in the six hours between 22 and 28 hours after isolation one-and-a-half

divisions occurred in the culture 237 A, yielding a total population of 20 individuals, and two divisions occurred in the culture 237 B, yielding a total population of 16 individuals. In the first 22 hours divisions occurred at the rate of one in seven hours in culture 237 A, and of one in 11 hours in culture 237 B, but in the succeeding six hours divisions occurred at the rate of one in four hours in culture 237 A and one in three hours in culture 237 B. Similar phenomena were exhibited, without any exception, by all of the cultures in which counts were made at several successive intervals of time.

The multiplication of infusoria, therefore, partakes of the autocatalytic character which has been found to distinguish multiplication of cells and growth in all of the various forms of life which have hitherto been investigated from this point of view. It must therefore be regarded as the expression of a fundamental property of protoplasm. Obviously it depends upon the contiguity of the cells to one another in a limited space and establishes the existence of a mutual influence upon one another's multiplication-rate which will be discussed more fully in the succeeding article.

The Influence of Temperature upon the Viability of Cultures and the Protective Effect of Mutual Contiguity.

The temperature of 30° C. prevents subcultures from multiplying, and the isolated individual almost invariably dies. The following are illustrative experiments:

Normal Hay Infusion.

Culture number	Age of parent culture	Temperature	Individuals in 24 hours
112 A	3 days	30°	0
117 A	3 "	32°.8	0
117 B	3 "	32°.8	0
125 A	3 "	29°.5	0
125 B	3 "	29°.5	8
152 A	2 "	30°	0
152 B	2 "	30°	4

Control cultures from the same parent cultures and kept at lower temperatures displayed normal reproductive capacity. Exactly the same phenomena were exhibited in bacterised infusions:

Bacterised Hay Infusion, unfiltered.

Culture number	Age of parent culture	Temperature	Individuals in 24 hours
153 A	2 days	30°	0
153 B	2 "	30°	0
156 A	4 "	30°.5	0
156 B	4 "	30°.5	0
157 A	2 "	30°.5	0
157 B	2 "	30°.5	0

Hence paucity or abundance of food does not influence the result. That true thermal death has occurred and not merely encystment is shown by the fact that these cultures never recover or become inhabited by infusoria after removal to a lower temperature.

This high degree of thermal sensitivity is very remarkable because shade temperatures above 30° C. are very usual during the summer months in South Australia, so that these organisms must frequently be under necessity of withstanding temperatures which, under the laboratory conditions specified, are invariably fatal. It was at first thought that the high degree of thermal sensitivity might be due to repeated culturing for very many generations under laboratory conditions, and to test this point "wild" individuals were obtained and cultured under identical conditions. All of the cultures exposed to temperatures above 30° C. died, while cultures into the same infusion at lower temperatures showed reproductive capacity similar to that of the laboratory strains.

It is possible that under conditions of simultaneous heat and desiccation viability at high temperatures may be increased by the assumption of a condition of arrested metabolism, *i.e.* encystment. But another possible means whereby "wild" infusoria of this species may be able to survive summer conditions is indicated by the fact that well-populated cultures are able to withstand much higher temperatures than isolated individuals. During the summer months well populated slide-cultures of the laboratory strains have successfully withstood exposure to room temperatures of 33° to 34° C. for from five to seven days in succession without death or any apparent injury or abnormality. Moreover subcultures propagated on slides by the introduction of 20 or 30 individuals into fresh hay infusion invariably survive and multiply at these temperatures, whereas single individuals, isolated from the same parent culture and habituated to the high temperature, invariably perish.

This result cannot be attributed to the introduction of greater numbers of bacteria together with the greater numbers of infusoria into the fresh hay infusion, because the viability of isolated individuals at high temperatures is not in any way increased by previous bacterisation of the infusion. Again we are led to infer the existence of a mutual influence, arising from the contiguity of cells, which in this instance is protective, enabling the organisms to withstand some adverse condition arising out of the high temperature of the environment which is fatal to isolated individuals.

The nature of the adverse agency at high temperatures is not yet ascertained. It is conceivable that it is a direct action of the temperature upon processes occurring in the organism itself, leading to the formation of toxic products of metabolism, to over-rapid metabolism, or destruction of essential enzymes, or, on the other hand, that the action of high temperatures is indirect, arising from the development of an adverse type of bacteria, or of an excess of certain bacterial products. The experimental evidence which is as yet available does not suffice to decide between these alternatives. But from whatever source the adverse effects of temperatures above 30° C. may arise, the protective effect of mutual contiguity of the organism remains unquestionable.

SUMMARY.

1. The rate of multiplication of single infusoria (*Enchelys farcimen* Ehr.) isolated from parent cultures of various ages falls off, at first very rapidly and later more slowly, with increasing age of the parent culture.

2. This loss of reproductive capacity has been shown not to be attributable to the accumulation of any retarding substances in old cultures, but to a period of existence at a low nutritive level.

3. Bacterisation of the hay infusions employed as culture media, by preliminary standing at room temperatures for from 24 to 72 hours, remarkably stimulates the reproductive capacity of the isolated organisms, and it delays, but does not prevent the loss of reproductive capacity which occurs with advancing age of the parent culture.

4. Heating the bacterised infusion to boiling-point, or even for 90 minutes on a boiling water-bath just before its employment, does not diminish the multiplication-rate of individuals isolated into it.

5. Although the final number of infusoria produced in a given culture is sooner or later determined by the number of bacteria available for food, yet the initial accelerative effect of bacterised infusions upon reproductive rate is not diminished by removal of the bacteria by filtration through a Berkefeld filter.

6. The accelerative effect of the bacterised infusion is not, therefore, dependent upon the presence of food, *i.e.* of bacteria, living or dead, but upon the presence of a soluble, non-volatile, thermostable product arising from the inhabitation of the medium by bacteria. It is proposed to distinguish this substance by the provisional designation of "X-substance."

7. The multiplication-rate undergoes a progressive increase with each successive division during the early stages of the development of a culture. Hence the multiplication of infusoria partakes of the autocatalytic character which has previously been shown to distinguish other types of growth, in higher plants and animals as well as in cultures of unicellular organisms.

8. This autocatalysis of multiplication is due to the contiguity of the cells to one another in a limited space, and is not merely the expression of the increasing abundance of foodstuffs due to the simultaneous multiplication of bacteria in the culture.

9. Temperatures above 30° C. prevent subcultures of single individuals of *Enchelys farcimen* from multiplying, and the isolated individual at this temperature almost invariably dies.

10. Cultures initially containing a large number of individuals, however, will multiply and survive continued exposure to considerably higher temperatures. Individuals isolated from such cultures and kept at the same temperature invariably die. Hence the contiguity of many organisms affords mutual protection against the adverse effects of high temperatures.

The expenses of this research were defrayed by the Animal Products Research Foundation of the University of Adelaide.

REFERENCES.

- Bachmann (1919). *J. Biol. Chem.* **39**, 235.
Eddy, Heft, Stevenson and Johnson (1921). *Proc. Soc. Exp. Biol. Med.*, New York, **18**, 138.
Emmett and Stockholm (1920). *J. Biol. Chem.* **43**, 287.
Engelmann (1876). *Morph. Jahrb.* i. 573.
Funk and Dubin (1920). *J. Biol. Chem.* **44**, 487.
Jennings (1910). *J. Exp. Zool.* **9**, 279.
—— (1913, 1). *J. Exp. Zool.* **14**, 293.
—— (1913, 2). *J. Exp. Zool.* **14**, 346.
Maupas (1888). *Arch. Zool. Exp. Gen.* (2), **6**, 165.
—— (1889). *Arch. Zool. Exp. Gen.* (2), **7**, 149.
McKendrick (1910). *Proc. Roy. Soc. Edinburgh*, 649.
Ostwald (1908). *Vorträge und Aufsätze ueber Entwicklungsmech.*, Heft 2.
Robertson (1908). *Arch. Entwicklungsmech.*, **25**, 581; **26**, 108.
—— (1913). *Arch. Entwicklungsmech.*, **37**, 497.
—— (1915). *Amer. J. Physiol.* **37**, 1 and 74.
—— (1916). *Amer. J. Physiol.* **41**, 535.
Williams (1919). *J. Biol. Chem.* **38**, 465.
Woodruff (1911). *Arch. Protistenkunde*, **21**, 263.