CLXII. ALLELOCATALYTIC EFFECT IN CULTURES OF *COLPIDIUM* IN HAY-INFUSION AND IN SYNTHETIC MEDIA.

By THORBURN BRAILSFORD ROBERTSON.

From the Darling Laboratories of Physiology and Biochemistry, University of Adelaide, South Australia.

(*Received August 25th, 1924.*)

In a recent paper [1923, 2] Cutler and Crump have described experiments in which from one to four individuals of *Colpidium colpoda* were isolated into varying volumes of a synthetic culture medium. Previous experiments by the author [1921, 2] had shown that when two individuals of another ciliate infusorian (*Enchelys*) are isolated together into a definite small volume of culture medium, the reproductive rate of both individuals is enhanced so that not merely twice, but from four to sixteen times as many individuals may be produced in 24 or 48 hours as in a culture of the same volume into which only a single individual was originally introduced. It was shown that the effect was not due to conjugation, because conjugation did not occur under the conditions which prevailed in these experiments, while if it had occurred, the result, as Jennings has shown [1913], would have been the opposite of the effect which was observed. This mutual acceleration of reproductive rate by contiguous organisms was termed allelocatalytic effect [1922], and from the autocatalysis which is known to occur during the growth of all organisms, it was inferred that this effect is common to all cells which live in proximity to one another in a limited volume of nutrient medium [1923].

Cutler and Crump, however, failed to observe allelocatalytic effect in their cultures of *Colpidium*. Two possible origins of this divergence of results at once suggested themselves. Either the infusorian employed by Cutler and Crump did not exhibit the effect at all or to a degree much less apparent than *Enchelys*, or else the exhibition of allelocatalytic effect is dependent upon some quality of the nutrient medium which was absent in the synthetic media which were employed by these investigators. The latter alternative appeared the more probable of the two, but in order to exclude definitely the former alternative, it was considered necessary to procure *Colpidium* and repeat the original experiments in hay-infusion medium with this organism and then, with both *Enchelys* and *Colpidium*, to repeat the experiments in the synthetic medium which was employed by Cutler and Crump.

It proved to be a matter of some difficulty to procure *Colpidium* in this locality and much time was consumed in a vain search for this organism. At length it was discovered that a ciliate infusorian closely resembling *Colpidium colpoda*, but considerably larger, is present in small numbers in fresh infusions of various plants obtainable here, but very rapidly disappears as the culture ages, its place being taken by a dense population of *Enchelys* and other ciliates. This *Colpidium* is somewhat longer than *Enchelys*, definitely kidney-shaped and, on the unindented side, it is nearly circular in outline, so that it is almost as broad as it is long, and its volume is therefore considerably in excess of that of *Enchelys*, while the variety of *Colpidium* employed by Cutler and Crump was considerably smaller than *Enchelys* both in length and in breadth and, moreover, was elliptical and not circular in outline. I have been unable to identify this organism more precisely. Unlike *Enchelys* it encysts very readily when deprived of food and from the cysts two or, more frequently, four daughter cells emerge. In the presence of an abundant food-supply, however, it divides, as *Enchelys* does, by transverse binary division.

This organism, *Colpidium* sp., although not identical with the species employed by Cutler and Crump, certainly belongs to the same or a closely allied genus, and it differs sufficiently from *Enchelys*, in morphological detail and physiological behaviour, to afford a satisfactory means of ascertaining the prevalence of allelocatalytic effect among different species of ciliate Infusoria.

The organisms were cultivated in hay-infusion prepared by heating 5 g. of chaffed oaten hay in 100 cc. of distilled water on a boiling water bath for half-an-hour. The filtrate, received into vessels which had previously been sterilised by steaming, was brought to various p_H values by the addition of small quantities of solutions of buffer salts. It was found that *Colpidium* would live and reproduce over a wider range of p_H than *Enchelys*. Satisfactory cultures were obtained in media containing 2 cc. per 100 cc. of $M/15$ phosphate mixture having a p_H of 7·7.

A small number of individuals contained in about 0·003 cc. of culture medium were removed from the parent culture by means of a capillary pipette and placed upon the depression in a slide. To this was added 0·1 cc. of fresh culture medium[1]. From this mixture a single individual was picked out in 0·003 cc. of fluid and isolated into 0·05 cc. of fresh culture medium. This organism was again isolated in 0·003 cc. of fluid and introduced into 0·05 cc. of fresh culture medium, forming the final culture. From the same parent culture two individuals were similarly isolated and re-isolated into a like volume of culture medium. The results were variable in magnitude but invariably of the same character. The reproductive rate of both individuals was much enhanced by their mutual contiguity and instead of twice as many

[1] This was aerated by passage of a stream of air, filtered through sterilised absorbent cotton, for from one-half to one hour before the buffer was added. The boiled hay-infusion does not contain sufficient oxygen for the requirements of the organisms and individuals isolated into unaerated culture media frequently die.

individuals being produced in 48 hours, from four to sixteen times as many individuals were produced in these cultures as in those which originally contained but a single individual.

The experiments were now repeated with one of the synthetic culture-media employed by Cutler and Crump [1923, 1]. This medium had the following composition:

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		

Solutions of the several constituents were prepared which were in each case ten times the final concentration desired. Ten cc. of each solution were then introduced into a 100 cc. graduated flask, a $M/15$ phosphate buffer added in sufficient amount to render the final $p_H = 7·4$ and the volume made up to 100 cc. by the addition of distilled water. The mixture was autoclaved on three successive days and allowed to stand thereafter for several days in a flask plugged with cotton before it was employed. It was not aerated.

Single individuals and pairs of individuals were isolated into this medium in the manner outlined above. The following are illustrative results:

Parent culture	Subculture	No. of individuals isolated into 0·05 cc.	No. of individuals in culture after 24 hours	48 hours
1 A (*Enchelys*)	5 A	1	3	30
1 A „	6 A	2	14	120
1 A „	7 A	1	3	24
11 A „	12 A	1	—	4
11 A „	17 A	2	—	11
1 B (*Colpidium*)	15 B	1	1 (dividing)	2
1 B „	16 B	2	7 (1 dividing)	18
11 B „	17 B	1	1 „	—
11 B „	20 B	2	4	—

Thus allelocatalytic effect occurs in cultures of *Colpidium* and *Enchelys* in the synthetic culture medium employed by Cutler and Crump, the number arising in 24 or 48 hours in cultures inoculated with two individuals being invariably in excess of twice the number produced in cultures inoculated with a single individual.

The discrepancy between Cutler and Crump's results and my own arises, therefore, neither from the difference of species nor from the nature of the culture medium employed by them. This fact suggested that the discrepancy arose from some detail of experimental technique and a further scrutiny of the methods employed by Cutler and Cramp ultimately revealed its origin.

The accelerative agent which is responsible for the increase of the division-rate of contiguous infusoria in a limited volume of culture medium issues from the cells only during the divisional period. This is shown by the fact that the period of "lag" is in no degree shortened by contiguity of other organisms. I have found, in cultures of *Enchelys* in hay-infusion, that whether one or fifty individuals are isolated into a given volume of culture medium, the period which elapses before the first division occurs, the "lag-period,"

is always the same. It varies with the age of the parent culture, lengthening, as I have previously shown [1921, 1; 1922], as the parent culture increases in density of population, but it does not vary with the number of individuals which are introduced into the subculture. Only after the first division has occurred does the allelocatalytic effect of the contiguous cells become manifest and the effect frequently increases for some time with successive divisions. In a culture containing 65,536 individuals per cc. (16 generations), therefore, the concentration of the catalytic agent will correspond to 65,535 cell divisions, which is the sum of all the divisions which have occurred during the production of 65,536 individuals from a single individual. If a small proportion of this culture be introduced into 100 times its volume of fresh culture medium, the concentration of the accelerative agent in this mixture will be the same as that in a culture in which 655 divisions per cc. have occurred, and if it be introduced into 1000 times its volume of fresh culture medium, the concentration of accelerative agent will still correspond to 65 divisions per cc. Now accumulation of autocatalyst sooner or later leads, as I have shown elsewhere [1922], to *slowing* of the reproductive rate and ultimately reduces it to zero. It is for this reason that the lag-period exhibited by individuals isolated from old parent cultures is so prolonged. In culture media which contain from the beginning such high concentrations of the catalytic agent, therefore, the addition of more by divisions occurring within the fluid, so far from being advantageous, might either leave the reproductive rate unaffected or even retard it. Cutler and Crump inoculated volumes varying from 0·37 to 9·8 mm.^{3} with 0·01 mm.^{3} of fluid obtained from the parent culture. The dilution of the autocatalyst originally present in the parent culture was therefore only from 1 in 37 to 1 in 980, whereas in my experiments, as a simple calculation shows, the dilution of the parent culture fluid is no less than 1 in 9000. After the first division, the concentration of autocatalyst in Cutler and Crump's cultures would rise to that corresponding to 3650 divisions per cc. in the cultures of smaller volume and to that corresponding to 165 divisions per cc. in the cultures of larger volume. In the cultures of smaller volume, therefore, the concentration of autocatalyst would rise very rapidly and soon attain the concentration at which its accelerative effect is replaced by retardation. That the reproductive rate of the organisms was actually retarded by the high concentration of the substances introduced together with the organisms into the subculture may be seen by comparing the average reproductive rates found by Cutler and Crump in their cultures of small and large volume respectively. The following table, compiled from their results, reveals very clearly the retardation which occurred in the culture of smaller volume into which a single individual was introduced:

Volume of subculture in mm.^{3}	Average reproductive rate in 24 hours
0 to 1·5	1·98
1·5 to 3·0	2·27
3·0 to 6·0	2·86
6·0 to 9·0	2·87

In cultures containing two individuals the concentration of autocatalyst would mount still more rapidly and the stage of retardation would be all the sooner attained. So far from obtaining allelocatalytic effect, therefore, either no mutual acceleration of reproductive rate or even retardation might be anticipated, and the results obtained by Cutler and Crump confirm this anticipation.

I have imitated the conditions which prevailed in Cutler and Crump's experiments by omitting the second isolation of the individuals inoculated into the drops forming the subcultures. From a parent culture of *Enchelys* which was four days old and thickly inhabited by infusoria, a small number of individuals were removed in about 0·003 cc. of fluid. This was diluted with 0·1 cc. of synthetic medium and from this mixture one individual was isolated into 0·05 cc. of the synthetic culture medium and two individuals were inoculated into an equal volume of the same medium. This procedure was then repeated, but the individuals now isolated were re-inoculated into 0·05 cc. of synthetic medium, so that while in the first case the substances originally present in the parent culture were only diluted to 1 in 555, in the second case they were diluted to 1 in 9259. The following were the results obtained:

No. of individuals inoculated	Treatment of the individuals inoculated	Individuals produced after 48 hours
1	Not washed by re-isolation	48
2	,, ,,	94
1	Washed by re-isolation	24
2	,, ,,	120

It is evident that allelocatalytic effect was totally abolished by the presence of even 1/555th of the concentration of the substances contained in the parent culture medium but was very marked when their concentration was reduced to 1/9259. The absence of allelocatalysis in Cutler and Crump's cultures, therefore, originated in the contamination of their subcultures by substances derived from the parent cultures. Had the organisms been washed by re-isolation then, as the above results show, allelocatalysis would have been observed[1].

There existed, however, another source of error in Cutler and Crump's experiments which might very readily have obscured the allelocatalytic effect, even if the organisms had been washed by re-isolation. They state that the individual, or individuals, which were inoculated into the subculture were "transferred by a capillary tube from the parent culture with about 0·01 mm.^{3} of liquid." In a previous paper they point out that the distribution of infusoria throughout their cultures was approximately uniform, so that a given fraction of the culture always contained, within narrow limits of error, the same

[1] In a previous paper it is pointed out that washing the infusoria in hyperalkaline distilled water (2 cc. of $N/10$ Na_2CO_3 to 100 cc. distilled water) abolishes the allelocatalytic effect. This is, however, not due, as was then supposed, merely to removal of associated bacteria or their products, but to some specific effect of the hyperalkaline distilled water, for no amount of washing in buffered hay-infusion will abolish the effect.

number of organisms. No details are given of the age or population of the parent cultures from which their subcultures were inoculated, but the data already quoted suffice to show that if in one case 0·01 mm.^{3} of the parent culture contained a single individual, and in another case two, then the population of the latter parent culture must have been greater than (approximately double) the population of the former. Now the lag-period varies with the population of the parent culture, increasing as the population increases, and therefore the lag-period, when two individuals were isolated, must have been longer than in those instances in which a single individual was isolated. The same considerations apply with proportionately greater force to those cultures which contained three or four individuals. If, now, the reproductive rate be estimated from the number of individuals produced in 24 hours, the lag-period will form a large proportion of that time, so that a more prolonged lag-period may offset the effect of an accelerated reproductive rate. Thus, suppose the interdivisional period to be 4 hours in the one case and 2 hours in the other, and the lag-period 16 hours in the former case and 20 hours in the latter. Then in 24 hours the numbers inhabiting the two cultures will be identical, although the reproductive rate in the latter case is actually double that obtaining in the former. This source of error was avoided in my experiments because the single and the paired individuals were isolated from the same parent culture and, as indicated above, the lag-period does not vary with the density of the initial population of the subculture. The number of individuals present after 24 hours must, in this event, be greater in the culture having the higher reproductive rate[1].

Cutler and Crump have repeated and confirmed my observation that cultures of infusoria contain a substance or substances capable of accelerating the multiplication of infusoria in scantily populated cultures, and they also show that similar accelerative substances may be extracted from the crushed bodies of infusoria. They find that the acceleration due to the addition of these substances to the culture medium is only exhibited after some delay and they state that "This is contrary to the findings of Robertson and is difficult to understand on the analogy of enzyme action." These authors are, however, labouring under a complete misapprehension of the facts, and it appears necessary to quote, therefore, the following remarks from my paper

[1] It was, however, for this reason that I was formerly led to report that allelocatalytic effect is diminished or absent when individuals from old parent cultures are inoculated together into the same drop [1921, 2]. Not only must the washing of individuals taken from old parent cultures be more thorough to remove the accumulated autocatalyst, but also allowance must be made for the fact that proportionately slight variations in the length of the lag-period will lead to great apparent differences of reproductive rate, if the lag period is long and included in the estimation of division-time. Obviously the effect of variations in lag, if they are not too great, will be minimised as multiplication proceeds and hence allelocatalytic effect which is not apparent in 24 hours frequently becomes quite evident in 48 hours. If these sources of error are carefully excluded, allelocatalytic effect is just as apparent in cultures inoculated with individuals derived from old parent cultures as it is in cultures inoculated with individuals derived from young parent cultures.

on "Reproduction in Cell-communities": [1922]: "Now I have repeatedly observed that the effect of accelerative agents upon the multiplication of infusoria is not manifested to any important degree during the period of lag.... The accelerative agent which is discharged by multiplying infusoria into buffered distilled water does not abbreviate the lag-period of infusoria isolated into nutrient media which contain it. Again, yeast extract shortens the lag-period only to a relatively insignificant degree while, subsequently to the first division, the reproductive rate is decidedly enhanced." Since the greater part of the first 24 hours after isolation of infusoria from a densely populated parent culture is consumed by the lag-period, it will be clear that Cutler and Crump's results are, in this particular, absolutely concordant with mine. Nor are they at all difficult to understand if one accepts their obvious meaning, that the accelerative agents concerned act upon the nucleus and cannot enter or leave the nucleus, except during the divisional period when the nuclear membrane no longer interposes a barrier between the nucleus and the cytoplasm which envelops it.

Greenleaf [1924], working with *Paramoecium* and *Pleurotricha*, has also recently reported failure to observe allelocatalytic effect in cultures of these organisms. While his account of his experimental technique is very meagre the origin of the negative results obtained in these experiments seems to be sufficiently obvious. He isolated single individuals into volumes of hay-infusion varying from two to forty drops and compared the populations attained after five days, from which the rate of multiplication per diem was computed. Now the maximal density of population in an infusorial culture does not vary greatly with the volume of the culture medium, provided, that is, that the infusoria originally inoculated into the culture are adequately washed to remove autocatalyst derived from the parent culture. Hence in twenty drops, for example, more divisions would be required to attain maximal population than in two drops. A simple calculation shows that if forty drops = 1 cc., then a maximal population of 65,000 individuals per cc. will arise from a single individual in from eleven to twelve generations in two drops of culture fluid, and not until sixteen generations in forty drops of culture fluid. In cultures of *Enchelys* and *Colpidium*, the maximal population of one or even two drops of culture medium originally inoculated with a single individual may be attained within three days if the lag-period is not extraordinarily prolonged. Hence in Greenleaf's experiments the cultures in the small volumes had ceased to reproduce some time before the count was made, while those in twenty drops may have been still dividing[1], and of course the rate per diem calculated from these populations appeared higher for the cultures of larger volume. In my experiments on the influence of culture volume upon the reproductive rate of isolated *Enchelys*, the initial or early rates of reproduction were compared and found to stand in nearly inverse proportion to the volume

[1] For, as Cutler and Crump have shown, the interdivisional periods are much more prolonged as the population approaches a maximum.

of the culture. It did not at that time appear to me to be necessary to point out that the superiority of reproductive rate in cultures of small volume could not possibly continue after, or even near, the attainment of maximal population. Greenleaf also failed to obtain allelocatalytic effect in cultures of equal volume containing one and two individuals respectively. Again, it appears necessary to draw attention to the obvious fact that two individuals isolated into a small volume of culture medium will produce a maximal population more quickly than one, and their progeny will, in consequence, cease multiplying while the progeny of the single individual are still undergoing reproduction.

Summary.

1. Cutler and Crump failed to observe allelocatalytic effect (the mutual acceleration of reproductive rate by contiguous organisms) in cultures of *Colpidium colpoda* in a synthetic medium.

2. The author has observed allelocatalytic effect in cultures of *Colpidium* sp. and *Enchelys* in hay-infusions and also in the synthetic medium employed by Cutler and Crump.

3. The failure of Cutler and Crump to observe allelocatalytic effect was due to the omission of the precaution of washing the organisms by re-isolation from a relatively large volume of culture medium before inoculation. It is shown that the dilution of the substances contained in the parent medium was only from 1 in 37 to 1 in 980 in the cultures employed by Cutler and Crump, whereas in the author's cultures it was 1 in 9000. It is shown that the concentration of parent culture medium contained in Cutler and Crump's cultures was sufficient to suppress the allelocatalytic effect and the cause of this suppression is explained.

4. In comparing reproductive rates in infusorial cultures the individuals inoculated into the cultures must all be derived from one parent culture, so that the lag-period is the same for all of them, or else the variable period of lag must be subtracted from the total period occupied in multiplication before the reproductive rates are compared.

5. In comparing reproductive rates in infusorial cultures care must be taken to estimate the population some time before it has attained its maximal density, for whatever the speed with which maximal population may be attained its final density in any given culture medium and at any given temperature is approximately the same.

References.

Cutler and Crump (1923, 1). *Biochem. J.* **17**, 174.
—— —— (1923, 2). *Biochem. J.* **17**, 875.
Greenleaf (1924). *Proc. Soc. Exp. Biol. Med.* **21**, 405
Jennings (1913). *J. Exp. Zool.* **14**, 293.
Robertson (1921, 1). *Biochem. J.* **15**, 595.
—— (1921, 2). *Biochem. J.* **15**, 612.
—— (1922). *J. Physiol.* **56**, 404.
—— (1923). The Chemical Basis of Growth and Senescence. (Philadelphia.)