CONJUGATION AND ENCYSTMENT IN DIDINIUM NASUTUM WITH ESPECIAL REFERENCE TO THEIR SIGNIFICANCE

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INTRODUCTION

That conjugation is a rejuvenating process of some sort or another has been very widely held. Engelmann ('76), Bütschli ('76), Maupas ('88 and '89), Hertwig ('89), Calkins ('06, '09, '13) and many others support this idea. Not all of these investigators agree with reference to the details concerning the rejuvenating effect, but practically all hold that if conjugation is prevented the race dies out, indicating that the progeny of a single individual passes through periods of vitality corresponding somewhat to youth, maturity and old age in metazoon individuals.

Calkins has very ably championed this view. He says ('09, p. 103) referring to the offspring of an exconjugant:

> If at any given period [we] could combine them in one mass of cells, we should have the analogue of a metazoon and would find that the protoplasm represented by the aggregate cells would manifest the same successive periods of vitality as those of youth, adolescence, and old age in Metazoa. We would find that the young cells divided more

rapidly than they do later in the cycle; we should find that after a certain time they become sexually mature and are able to conjugate and so to perpetuate the race; and we would find that, ultimately, evidences of weakened vitality and degeneration appear in the aggregate of cells, and that they finally die of old age.

Calkins consequently holds that conjugation causes an increase in the rate of fission and he supports this view by experimental evidence which will be presented later. In this contention, however, Calkins stands practically alone. Neither Maupas nor Hertwig, both of whom carried on very extensive experiments on various protozoa, were able to find any evidence indicating an increase in the rate of fission after conjugation. In fact, Hertwig actually found a decrease. But both found in their pedigree cultures that the race died out after a certain number of vegetative divisions and they maintain that this must have been due to the absence of conjugation. Consequently they concluded that conjugation, although it does not cause an increase in fission-rate, is a rejuvenating process which prevents the race from dying out.

This view received very serious opposition in the results obtained by Woodruff in an extensive series of experiments on Paramecium ('11, '12, '13, '14). These results prove conclusively that certain races of Paramecium can continue to reproduce indefinitely without conjugation.

Jennings has recently ('13) with characteristic thoroughness, investigated the problem of the significance of conjugation in Paramecium. He maintains (p. 293) that "Conjugation decreases the rate of fission, causes a great increase in variation in the fission rate, brings about many abnormalities, and greatly increases the death rate." And he concludes (p. 378)

Conjugation does not produce rejuvenescence, for after conjugation most of the animals are less vigorous than before. *What conjugation does is to bring about new combinations of germ plasm, just as is done in the sexual reproduction of higher animals. One result of this is to produce biparental inheritance; another is to give origin to many variations; in the sense of inherited differentiations between different strains. Some of the new combinations are better adapted to the existing conditions than others; these survive while the others die out.*

Thus we find that Maupas obtained no increase in the rate of fission after conjugation and that both Hertwig and Jennings obtained a decrease. Calkins however still retains his original idea regarding the effect of conjugation on the rate of fission. He says ('13, p. 523)

> Experiments here re-described show that the vitality of a given race is increased by conjugation. An ex-conjufant from a pure line that had lived for 369 generations in culture, divided 376 times after conjugation in nine months, while the pure line control that had not conjugated, and from which the ex-conjugant was obtained, divided only 277 times in the same period.

Following Fermor ('13) Calkins has recently ('15) extended to encystment the idea that conjugation causes an increase in the vitality of the race. He maintains that there are two kinds of cysts, one kind serving merely to tide over periods of unfavorable environmental conditions, the other serving in the process of nuclear reorganization, structurally and physiologically. Thus he maintains that encystment at times has essentially the same function as conjugation, that it is a rejuvenating process resulting in an increase in the rate of fission. He presents experimental evidence in support of this contention and maintains that it is also supported by the discovery of Woodruff and Erdmann ('14) that in Paramecium reproducing vegetatively there are periods of depression ending in nuclear reorganization which is followed by increased vigor.

The experiments described in this paper were continued, with some intermissions, from April, 1910 to May, 1914. They were undertaken primarily to ascertain for Didinium, the relation between conjugation and the rate of fission and to ascertain in general the effect of preventing conjugation. Aside from the evidence bearing directly on these problems there was, however, considerable evidence obtained which has important bearings on other problems, notably, the function of encystment, vitality of cysts, mutations, the relation between conjugation and death-rate, and variability in the rate of fission. Questions concerning the vitality of cysts and mutations will be discussed in separate papers, which will appear elsewhere; all of the other problems will be considered in the following pages.

MATERIAL AND METHODS

Throughout the entire period covered by the experimental work connected with this paper numerous observations were made on didinia collected in various places and on mass cultures kept in the laboratory under various conditions. These observations concerned primarily the life history and the general physiology of the organisms, especially those phases of it which are associated with the processes of encystment and conjugation and the relation between these processes and the environment. The main part of the experimental work consisted, however, of the study of a series of groups of pure lines which were started and maintained as follows.

Four pairs of conjugating didinia were taken from a vigorous culture which had been in the laboratory for some time Each of these four pairs was then put into a rectangular watch-glass containing a few drops of solution and a considerable number of paramecia The watch-glasses were now piled one upon the other, placed into a damp chamber and later examined daily. Six of the ex-conjugants died within a few days. The other two divided and from each, two lines were started and labeled (conj.) indicating that conjugation had occurred. Thus there were four lines started with individuals which had conjugated. Simultaneously four lines were started with individuals taken from the same culture, but which had not conjugated. From this group of lines other groups were started from time to time as indicated in table 1.

All of the cultures were kept continuously in the same damp chamber and all were treated as nearly alike as possible They were fed throughout the entire experiment practically exclusively on paramecia, which were obtained from four cultures kept in liter jars These cultures were continuously maintained in the most uniform and vigorous condition possible During the early part of the experiment one-half of the solution in each of the four jars was replaced by standard timothy hay solution (100 cc. water + 1 gram hay, boiled ten minutes) at given intervals but that in each jar at a different time. During the latter part the hay and water were added directly at various intervals depending upon the rate of development. This method was found to maintain the cultures more nearly in a uniform condition than the one first used. Moreover, in feeding the didinia, an equal amount of solution was always taken from two or more of the paramecia cultures and mixed in a separate dish, thus increasing the uniformity of the culture medium. Two drops, sometimes three, of this mixture which contained numerous paramecia were put into each of the required number of clean watch-glasses. A drop of solution containing one didinium was then taken from each of the pedigree cultures and put into each of the watch-glasses supplied with fresh food. After recording the number of fissions the remaining didinia were either destroyed or used in making further studies of conjugation and encystment. Nothing was sterilized in these experiments but the same pipet was used in transferring all of the didinia. Moreover, from time to time a drop of solution was taken from each of the pedigree cultures mixed and then a drop of the mixture added to each of the new preparations. Thus all were inoculated with the same bacteria and all were the same in other respects.

Throughout all the experiments the cultures were transferred and the number of generations recorded daily, twice a day, or every other day. During a greater part of the summer, when it was relatively hot, the fission-rate was so high that it was found advantageous to transfer twice a day, for eight or even nine generations, over 1000 individuals, were sometimes produced in twenty-four hours. During the rest of the year they were usually transferred daily. They were transferred every other day only during a few short periods, when the temperature was exceptionally low.

We shall present the results obtained in the experimental observations under several headings, as follows.

NATURE AND CAUSE OF ENCYSTMENT, VITALITY AND ACTIVATION OF CYSTS

When didinia are about to encyst they become quiet, loose their cilia, mouth and seizing apparatus and secrete a heavy wall about themselves. The wall usually adheres firmly to the substratum. Thus the cysts become fastened to various objects usually near the surface of the solution in which they live. Under certain conditions practically all of the didinia in a solution suddenly encyst, so that cultures having countless numbers of active didinia at a given time may have practically none a few hours later.

Encystment can usually be induced at any time by cutting off the food supply. But it frequently occurs when there is an abundance of food present and sometimes it does not occur when there is none. This is especially true for cultures in which conjugation has been prevented for a considerable number of generations. In fact, in lines which had passed through 1500 to 1600 generations it was almost impossible to induce encystment.

To induce the didinia to come out of the cysts it is usually only necessary to add a vigorous culture of paramecia, or the solution from such a culture. The paramecia are not absolutely necessary. Active didinia are ordinarily found twenty-four hours after the solution has been added; but at best only a very small percentage of the cysts develop. Some can, however, live a long time and drying under atmospheric conditions is not fatal. In an experiment described elsewhere, cysts kept sealed air-tight in a 10 cc. vial for nearly five years were still found to be viable.

Didinia are consequently very hardy and they are, as far as known, destroyed by only two organisms; a small rhizopod which probably develops inside of the cysts, devours the protoplasm and then bores its way out through the wall and a hypotricate which swallows them whole in considerable numbers.

Encystment clearly serves to bridge over unfavorable environmental conditions, and it unquestionably facilitates distribution, especially when the cysts are dry. Calkins maintains that it also functions as a rejuvenating process. Our results do not, however, support this contention, as will be demonstrated later.

NATURE, CAUSE AND DURATION OF CONJUGATION

Didinia that are about to conjugate divide in rapid succession two or three times without taking any food. During this process they become much reduced in size; the protuberance at the anterior end bearing the mouth becomes considerably enlarged and somewhat flattened; and when they are ready to conjugate they have a peculiar jerky movement. Such individuals are consequently readily recognized. They are usually found in restricted areas in the cultures. In watch-glasses having numerous didinia fairly uniformly distributed, I have repeatedly seen numerous individuals, some conjugating and others ready to conjugate, all crowded together in one small spot and none elsewhere. Whether these aggregations are due to a restriction of the preliminary processes to specimens in these regions or to reactions in the preconjugants which result in the aggregation, I am unable to say. However, the fact that they tend to remain in the region seems to indicate that they aggregate. They probably secrete some sort of substance, when they are in the conjugating state which acts on them as a weak acid acts on paramecia, but which does not affect individuals which are not ready to conjugate. The preconjugants in the restricted area swim about rapidly, continuously stopping and turning in various directions. Thus they frequently collide and if in these collisions they chance to meet mouth to mouth, they usually remain united owing to the adhesive character of the mouth during this period. Frequently the mouth of the two conjugants is not directly opposite and occasionally three individuals become united. Thus we see in these simple creatures, special provisions which facilitate the unions of sexually mature individuals, provisions which are in some respects similar to those found in the higher animals.

At first union is very weak so that the conjugants can readily be separated by squirting them out of a pipet. Later they become so firmly united that they cannot be separated without injury. The conjugating pairs continue to swim about, but their movements are, owing to their relative position, necessarily more or less uncoördinated. Usually one proceeds forward for a time and then the other. Thus they rarely get any considerable distance from the place where union occurred. The duration of the union varies greatly. It depends largely upon the temperature. Occasionally, pairs are found which never separate. But at room temperature, they usually remain united only about twenty to thirty hours. During this time there is nuclear transfer similar to that found in Paramecium (Prandtl, '06). All of the steps involved in this process, degeneration of macronucleus, divisions of micronucleus, etc., occur after the union of the conjugants takes place. Whether or not there is anything in the nature of nuclear reorganization in conjugants which are artificially separated is not known.

The ex-conjugants usually begin to feed about one hour after they separate. Then, after growing rapidly for about two hours, they divide, after which they proceed as usual. If they are not allowed to feed after conjugation occurs they do not divide. Nor do they encyst. Some of them live a week or more but all invariably die without fission if they are not fed. This was repeatedly observed and it was found to hold without exception.

The discovery that ex-conjugants die if they are not fed greatly facilitated the process of continuing, for a long time, pedigree cultures without conjugation; for such cultures were at different periods maintained in the encysted state, and it was often difficult to obtain cysts without at the same time having conjugants which were difficult to eliminate before it was known that they die if they are not fed.

The elimination of food, subjection to optimum temperatures and the presence of numerous individuals in a small space facilitate conjugation just as it does in Paramecium (Jennings, '10). This process, however, often occurs in the presence of much food with very few (4 to 8) individuals present, and it frequently does not occur in the absence of food, no matter how many are present or what temperature is employed. In fact I was quite unable in many instances to induce conjugation It is evident, therefore, that conjugation depends upon internal as well as upon external factors, but what these factors are is as yet unknown. The results of my experiments seem to indicate that conjugations can be induced more readily in lines in which it has been prevented for long periods than in lines in which it has recently occurred. They indicate, however, that there is no specific relation between the tendency to conjugate and the number of consecutive asexual generations produced in a line; for in several instances I obtained conjugation in abundance in lines which had produced less than 100 consecutive asexual generations and in others I was unable to induce conjugation in lines which had produced more than 1000 such generations. Moreover, in certain lines in which conjugation occurred among the accessory specimens at a given time it was not possible to induce conjugation during certain later periods, i.e., after more asexual generations had been produced. Furthermore, Calkins ('15, p. 238) obtained conjugation in individuals which had produced only two asexual generations; and Jennings ('10) obtained similar results in Paramecium.

THE EFFECT OF CONJUGATION AND ENCYSTMENT ON THE RATE OF FISSION

All of the evidence obtained on the relation between fission-rate and conjugation and encystment is presented in table 1. This table contains a brief history of all of the groups of pure lines that were studied. In it are given the time each group was isolated in relation to the time of conjugation and encystment, the ancestry and duration of each, the number of lines each contained and the average fission and death-rates.

An examination of table 1, with some explanatory additions, shows the following: In April, 1910, two groups of four lines each were isolated, one immediately, the other some time after conjugation. After these two groups had continued for thirty days the latter was closed. During this time the fission-rate in the conjugating group was slightly lower than it was in the non-conjugating group and the death-rate was also somewhat lower. On May 20 all of the dishes containing the didinia belonging to both groups were returned to the damp chamber after removing one specimen from each to continue the lines. In all of the dishes returned the didinia soon became very numerous and two days later conjugation occurred in one dish of each group. Fifteen conjugating pairs (30 individuals) from the non-conjugating group were isolated. Twelve of these individuals died without dividing. From the remaining individuals, four were selected to start a new group of five lines as represented in the table. During the next ten-day period the rate of fission in these lines, which had just conjugated was somewhat lower than it was in those which had conjugated forty days earlier, but during the following period it was a trifle higher. At the close of this period, June 11, all of the didinia were left in the dishes. They became very numerous and soon consumed all of the paramecia after which many of them encysted. The dishes containing the cysts were then properly labeled, sealed with paraffin and vaseline and stored away in the damp chamber. Toward the last of April, 1911, vigorous paramecia cultures were added to all of the dishes, and the following day active didinia appeared in one belonging to the more recently conjugating group. None appeared in any of the other dishes.

TABLE 1

Effect of conjugation and encystment on fission-rate and death-rate in Didinium

The large numbers in the columns represent the total average number of fissions in a group of lines for the period given above. The small numbers directly below the large ones indicate the number of lines in the group present at the beginning of the period; wherever these are missing the number of lines in the preceding period has been maintained. The small numbers followed by 'd' indicate the number of lines which died out and those followed by 'c' the number which encysted during the period. The brackets and arrows indicate the origin and ancestry of the different groups of lines. *Conj.*, *Conj. sep.*, *small*, *large*, signifies that the numbers following each refer to a group of lines which originated respectively from ex-conjugants, conjugants separated before nuclear transfer occurred, small individuals ready to conjugate, and large individuals not ready to conjugate. *Cyst.* signifies that the group of lines represented by the preceding numbers were encysted during the period indicated above.

The didinia soon became numerous and in a few days many conjugating pairs were discovered. In the meantime, they had been examined at intervals short enough to make it certain that conjugation had not previously occurred. Similar precautions were taken throughout the entire series of experiments.

From the culture containing the conjugating specimens five groups of lines were started May 2, 1911, as indicated in the table. The lines in two of these groups contained individuals which had conjugated normally (ex-conjugants); those in one group contained individuals which had been mechanically separated immediately after they had joined in the process of conjugation (conjugants separated or 'split pairs'); those in another group contained individuals which showed by their size and action that they were ready to conjugate (small); and those in the fifth group contained individuals which showed no indication of preparation for conjugation (large).

The table shows that these five groups in the beginning consisted respectively of 70, 20, 20, 13 and 10 lines, and that during the first ten-day period the fission-rate was somewhat lower in the two conjugating groups than it was in the three non-conjugating groups but that for the following three periods it was remarkably nearly the same in all.

At the close of these periods all of the active didinia died owing to excessively hot weather during which the temperature reached 35° several times. Cysts, however, which had formed in some of the dishes retained for special study after isolating individuals for propagating the lines, were not injured, and from one of these, four new groups of lines were started.

Thus, the experiment was continued, groups of new lines being started from time to time, some from individuals which had recently conjugated, some from conjugants separated, somo from individuals ready to conjugate, some from large specimens which were not ready to conjugate and some from specimens which had been encysted for various periods of time, as indicated in the table. Consequently there were present throughout the entire experiment continuously, several groups of lines which differed markedly with reference to the number of generations since encystment or conjugation had occurred in them. And since these groups all originated in the same individual and were carried along parallel with each other and treated alike, it is obvious that the rate of fission ought to differ in them if conjugation or encystment has any effect on it.

The detailed description, given above, of the earlier periods shows that if conjugation had any effect on the rate of fission during these periods it was a retarding effect which, however, continued for but a short time after conjugation. An examination of the table shows that the same may be said regarding all of the other periods. It shows also that encystment has no appreciable effect on the rate of fission. Take for example, the five day period (9/22–9/26, 1911) or better still, the twenty day period (9/7–9/26, 1911); here we find that the seven groups of lines produced during this period the following average number of generations respectively: 76, 75, 74, $74\frac{3}{4}$, 76, 73, $75\frac{2}{3}$, a maximum difference of only three divisions in twenty days, while the number of generations since conjugation had occurred in these groups of lines varied from 118 to 524 and the number of generations since encystment from 75 to 236. Moreover, the rate of fission is not greatest in the groups in which the period considered is nearest the point of conjugation and encystment or least in those in which it is farthest from these points.

The sixty day period immediately following (6/1–7/30, 1912) shows even a more striking similarity in the rate of fission in lines which have conjugated compared with those which have not. Here we find that a group of lines in the sixty days immediately after conjugation produced an average of 248 generations and that during the same period lines from the same stock but in which conjugation was prevented produced an average of $249\frac{3}{5}$ generations. Again, near the close of the experiment we find that in a twenty day period (5/9–5/29, 1913) lines immediately after conjugation produced 32 generations, while the stock from which they were isolated, after having passed through over 1,500 generations without conjugation, produced in the same period and under identical conditions, $33\frac{3}{5}$ generations. There is consequently no evidence whatever indicating that conjugation causes an increase in the rate of fission in accord with Calkins' contention and the same may be said with reference to encystment.

Calkins maintains that in Didinium the rate of fission is relatively high immediately after encystment; that after continuing for about 100 generations it becomes lower and continues to decrease for 30 to 50 generations when encystment again occurs, during which there is a nuclear reorganization resulting in rejuvenescence and an increase in the rate of fission. There is not the slightest evidence indicating that anything of this sort occurred in our experiment. If encystment affects the rate of fission there ought to be some evidence of it in the periods following (9/6, 1911) but there is none, as an examination of the results presented in the table for these periods clearly shows. Moreover, at the close of the last part of the experiment, three groups of lines had passed through 1035, 831 and 850 generations respectively, without encystment or conjugation, proving conclusively that if there are cycles with reference to encystment in accord with Calkins' contention, they are very different from those he describes.

It is true that toward the close of the experiment, after a long period without conjugation or encystment, the didinia seemed to lose their accustomed vigor, but neither conjugation nor encystment served to remedy matters as a comparison with the rate of fission in the wild groups during these periods shows. In reference to this the following extract taken from my notes is illuminating. It refers to the last periods of 1913, and gives a good idea of the condition of the didinia toward the close of the experiment.

All individuals not used to continue the pedigree lines were retained in their respective dishes. These were all examined from day to day. Conjugation occurred very freely in all but the wild lines, encystment very rarely. The didinia in all of these cultures eventually died out except a few cysts. Conjugation did not save them. Paramecia from five different cultures were tried as food also some paramecia fed on malted milk but the didinia still died. The individuals in the old lines were much smaller than those in the wild lines, the cysts were also relatively small and there was a strong tendency to produce monsters. There was no observable difference regarding this between the lines which had passed through 1559 generations and those which had passed through only 850. Various means were employed in attempting to induce encystment, but except in the wild lines in which it occurred freely, only a few were secured, none at all in the 850 generation group.

All of the cysts produced in the old lines were carefully preserved. The following year vigorous cultures of paramecia were as usual added to all of the dishes. Active didinia appeared in only one of twelve dishes containing cysts of the old culture, while in those containing the cysts of the wild culture, treated precisely the same, they appeared in practically all. From the didinia thus obtained two new groups of lines were started as indicated in the table. In both groups the rate of fission and the condition of the didinia was practically as it had been before encystment. There was no appreciable improvement in the old culture. The death-rate was not very much higher in this than it was in the culture from the wild race but the fission rate was much lower and there was no noticeable change in the condition of the specimens produced. These lines were carried along for forty days and then abandoned. They did not die out, but it is, of course, impossible to say whether or not they would have recuperated, if the experiment had not been closed.

As stated above, individuals recently isolated from a wild culture were much more vigorous than those in the lines long continued without conjugation, but the individuals in lines started from another wild culture were even less vigorous. These lines continued from 1/17 to 5/29, 1913. By referring to table 1 it will be seen that in them the fission-rate was considerably lower and the death-rate was much higher than it was in the lines long continued without conjugation, and that they died out at the close of the period, owing to the fact that it was impossible to induce them to encyst.

Thus we see that in these experiments there is no support for Calkins' contention concerning the effect of conjugation and encystment on the rate of fission. Nor is there any support for the contention of Maupas and others that protozoa must conjugate from time to time in order to continue the race, that is, that there are cycles consisting of vegetative and sexual reproduction alternating; for an organism that can produce 1646 vegetative generations can in all probability continue reproducing asexually indefinitely. There is, in fact, no evidence of cycles anywhere in the entire experiment. Toward the close there was to be sure a loss in vigor, but the loss was quite as great in the group of lines which had produced only 850 generations as it was in the one which had produced 1559 generations.

But if conjugation and encystment do not cause an increase in the rate of fission, how can the following results obtained by Calkins be explained? And if conjugation is not a rejuvenating process, what is its function?

Calkins in 1904 cultivated on slides a line of paramecia for 369 generations; then he started a new line from some of the individuals of this line, after they had conjugated, and carried it along parallel with the old line for nine months. During this time the former (ex-conjugant) produced 396 generations and the latter (non-conjugant) only 277 (Calkins and Gregory, '13, p. 517).

These results seem strongly to support Calkins' conclusion that conjugation causes an increase in the vitality of protozoa. The support is, however, not so strong as it seems. In the first place, both lines were not treated identically. "The ex-conjugant was treated for twenty-four hours with beef extract on December 9th, the A series (non-conjugant) with beef extract on December 14th, January 8th and 15th." It is not possible to say what effect this different treatment may have had, and it is, therefore, evident that it may have caused the observed difference in rate of fission. In the second place, Calkins and Gregory maintain that in different lines originating in the same individual there is a marked difference in the rate of fission. They say ('13, p. 477) concerning four such lines, A, B, C and D "In one hundred days a typical representation of A would have divided 65 times, of B, 90 times, of C, 81 times and of D, 95 times." Accordingly, if D had produced 396 generations, the number produced by Calkins' ex-conjugants mentioned above, A would have produced only 270.9 generations, that is, six generations less than Calkins' non-conjugants. It is consequently evident that here greater variation was found in four non-conjugant lines than was found in the ex-conjugant and non-conjugant lines mentioned above. Obviously then, the fact that the rate of fission differed in these lines adds extremely little support to the conclusion that conjugation causes an increase in vitality in protozoa.

Concerning the significance of the evidence which Calkins presents in support of the contention that encystment at times causes an increase in the rate of fission, the following may be said: Calkins kept didinia continuously in spring water which was frequently changed. To this he added from day to day a few paramecia. He maintains that, under such conditions, the rate of fission decreased while the rate of encystment increased until after the production of 84 to 148 generations all were encysted. He then poured off the old water and added "fresh water and Paramecium." This caused the didinia to come out of the cysts after which, he maintains, the rate of fission was again practically the same as it had been in the beginning. And he holds that this shows that encystment results in rejuvenescence.

The result of Calkins' experiments seem to show fairly clearly that there is a decrease in the rate of fission under the conditions stated and that there is an increase after encystment, but they do not seem to me to show that this increase is necessarily due to encystment. To recover the race from encystment Calkins, as stated above, replaced the solution by fresh water (he does not say what kind) and paramecia (presumably many). Thus he subjected the organisms to a marked change of environment. Now, Calkins and others have shown that just such changes cause definite increase in the rate of fission in Paramecium under conditions similar to those in which the didinia were before encystment. Is it not therefore reasonable to assume that the increase in the rate of fission in Didinium noted by Calkins was due to the change in the environment rather than to encystment? At any rate, we have demonstrated that Didinium can produce over 1000 generations without any apparent periods of depression except at the close and that after having produced many generations thus it was almost impossible to induce encystment. Calkins obtained even a more marked effect of continued culture on encystment. He says ('16, p. 266): "In my experiments with Didinium the race apparently lost its power to encyst and ultimately died out after six months' culture without encystment." If there really are in Didinium, cycles ending in encystment as Calkins maintains, they are very long and if encystment results in better adjustment to the environment during periods of depression, if it is a rejuvenating process, why was there not a strong tendency to encyst near the close of our experiment when the vitality of the didinia was low, instead of no such tendency whatever?

Calkins also obtained other results which do not seem to be in harmony with his conclusions. He says ('15, p. 238):

> During the first cycle no conjugating pairs were observed in any of the stock dishes although such material is prepared daily and always watched for at least five days. During the first week of the second cycle, epidemics of conjugation appeared in the stock dishes. This period of conjugation lasted about ten days, after which not a pair was seen. Conjugation epidemics appeared again in the third cycle and at a corresponding time. The first pairs were seen in the stock dishes on the third day after recovery from encystment (March 12) and pairings occurred in great numbers until March 20th, after which not one pair could be obtained from the material. During the height of the epidemic in the stock material two cases of conjugation occurred in the isolation cultures. One of these pairs (March 16) was the union of two individuals out of eight derived from one individual isolated the day before. The second case occurred on March 17 between two individuals among sixteen derived from an individual isolated the day before.

In the first cycle referred to the didinia produced 131 generations and then encysted; in the second they produced 148 generations. In each case conjugation occurred shortly after the didinia came out of the cysts. In my work I observed repeatedly a strong tendency to conjugate shortly after encystment, thus confirming the results obtained by Calkins. But if encystment and conjugation have the same function, if both are rejuvenating processes, why was there such a strong tendency to conjugate soon after "recovery from encystment?" And why was there no tendency to conjugate later in the cycle, especially at the close when all of the individuals encysted?

An examination of table 1 shows that in Didinium there was great variation in the rate of fission in different periods. These variations were largely due to changes in temperature. Woodruff maintains that there are similar variations in the rate of fission in Paramecium, but he holds that there are certain fluctuations in this rate in the absence of any variation in temperature or other environmental factors. These he calls rhythms. He says ('11, p. 353): "[Rhythms] are due to fundamental factors in cell phenomena and not to extraneous causes." For all that is known to the contrary there may have occurred in Didinium variations similar to those called rhythms by Woodruff. But if there were any such fluctuations they were either exceedingly small or they occurred simultaneously in all of the lines running parallel, regardless as to the difference in the number of generations produced by these lines since conjugation or encystment had occurred. Consequently, if there were such fluctuations of any considerable magnitude they could not have been specifically related to conjugation or encystment with reference to time.

By referring to table 1, it will also be seen that in 1912, 7/15–7/20, one of the groups of lines divided into two groups having different rates of fission. These two groups of lines originated from a single individual during a period of abnormally high temperature. They were carried for 315 days during which time one group produced a total average of 838 generations ($2\frac{2}{3}$ per day) and the other a total average of 634 (2 per day). The difference in the two groups was practically the same throughout the whole period and it appears to have been permanent. The origin and significance of this mutation will be more fully discussed in a separate paper.

As indicated in the table the pedigree culture continued with certain intermissions from April, 1910 until May 1914. During this time there were produced in one of the groups of pure lines an average of 1646 generations without conjugation and in the same group of lines 1035 generations without encystment. The stock became very weak toward the close but it did not die out, and, of course, it is not known how much longer it would have survived. The fact that it continued so long without conjugation or encystment seems to indicate that neither of these processes is necessary for continued existence.

This conclusion is in perfect harmony with what is known regarding the life-history of many of the lower plants and with the results obtained by Woodruff in very thorough and extensive experiments on Paramecium. Woodruff's conclusion is, however, open to criticism. He says, after having obtained 3029 generations without conjugation ('12, p. 123): "I believe this result proves beyond question that the protoplasm of a single cell may be self-sufficient to reproduce itself indefinitely, under favorable environmental conditions, without recourse to conjugation, and clearly indicates that senescence and the need of fertilization are not primary attributes of living matter." That the protoplasm of certain single cells is self-sufficient to reproduce itself without recourse to conjugation has, in my opinion, long since been known, for nothing in the nature of conjugation has ever been discovered in bacteria and in a considerable number of algae. If this is true, Woodruff's results merely support a well established conclusion regarding the necessity of conjugation for the continued existence of protoplasm; they make it possible to add Paramecium to the list of organisms in which conjugation has been found not to be necessary for continued reproduction.

THE EFFECT OF CONJUGATION ON DEATH-RATE

Jennings ('13, p. 293) found, in extensive and very thorough experiments with Paramecium that "Conjugation decreases the rate of fission, causes a great increase in variation in fission rate, brings about many abnormalities, and greatly increases the death-rate." He concludes (p. 378), as previously stated, that conjugation does not produce rejuvenescence but that it functions in the production of new combinations which are better adapted to the existing conditions than others and that these serve to perpetuate the race while the others die out.

The results obtained in our experiments with Didinium do not support these contentions. We have already demonstrated that conjugation in Didinium causes only a slight decrease in the rate of fission, if any at all, and in this and the following sections

TABLE 2

The effect of conjugation on death-rate

Conjugants separated signifies that conjugants were mechanically separated before fertilization; *small* signifies that the individuals were ready to conjugate; and *large* that they were not ready to conjugate. The numbers in all cases refer to individuals, not to pairs. The number died includes all individuals which did not divide after conjugation or after they were isolate, in case there was no conjugation.

DATE	EX-CONJUGANTS		CONJUGANTS SEPARATED		SMALL NON-CONJUGANTS		LARGE NON-CONJUGANTS	
	Number isolated	Number died	Number isolated	Number died	Number isolated	Number died	Number isolated	Number died
1910, 2/28	12	11						
1910, 4/17	8	6						
1910, 4/25	40	29						
1910, 5/23	12	5						
1911, 2/10	14	6						
1911, 4/30	70	48						
1911, 5/1	20	12	20	12	15	9	10	0
1911, 6/18	54	11	36	19	10	5	8	3
1911, 6/27	36	2	20	0			5	0
1911, 7/29	20	0						
1911, 8/2	24	9	2	1	5	0		
1911, 8/10	24	24					5	0
1911, 8/26	2	0	6	0			3	0
1912, 6/3	44	0	22	0			5	4 encysted
1912, 6/6	20	0	28	0			5	4 encysted
Total......	400	163	134	32	30	14	41	3

it will be demonstrated that it causes no appreciable increase in variation in the rate of fission or in death-rate.

The results obtained on the relation between conjugation and death-rate are, as previously stated, presented in brief form in table 1. A portion of these results is given in detail in table 2. This table contains a record of all isolated ex-conjugants which died without fission and of all other isolated specimens which died during the same periods. By referring to the table it will be seen that a total of 400 individuals which had conjugated were isolated and that 163, or 47.5 per cent of these died without fission; that a total of 134 conjugating individuals in which nuclear transfer was prevented were isolated and that 32, or 23.8 per cent, of these died without fission; that a total of 30 small individuals ready to conjugate were isolated and that 14, or 46.6 per cent of these died; and that a total of 41 large individuals not ready to conjugate were isolated and that only 3, or 7.3 per cent of these died.

These results show that the death-rate in the individuals not ready to conjugate was very much lower than in those ready to conjugate and they also show that the death-rate in the ex-conjugants taken as a whole was considerably higher than it was in the separated conjugants and those ready to conjugate. A further study of the table shows, however, that conclusions based on a consideration of the experiments taken as a whole are misleading. This is due to the fact that in a number of experiments in which only ex-conjugants were isolated, the death-rate was on an average higher than in the others. It is obvious, therefore, that these experiments should be omitted in the calculations. When this is done it leaves a total of 200 ex-conjugants of which only 34, or 17 per cent died without fission. That is, 5.8 per cent less than the percentage of deaths in the conjugants which were separated without nuclear interchange and 26.9 per cent less than that in the small individuals ready to conjugate. These experiments, therefore, indicate that conjugation produces a decrease rather than an increase in death-rate. They indicate, however, that the preliminary steps in the process of conjugation do cause an increase in death-rate, for in the large specimens in which no preparation for conjugation had occurred the death-rate was much lower than it was in those in which such preparations had occurred.

In the ex-conjugants and the small individuals ready to conjugate or separated immediately after conjugation the variation in death-rate was very large. In some experiments all died, in others none. In the large individuals not ready to conjugate it was much smaller. I believe the great variation in the death-rate of the former was largely due to the fact that in the process of isolation the individuals were transferred to solutions consisting of various degrees of dilution of the culture solution in which conjugation occurred. The results of observations made on this point, seem to show that the less the dilution of the solution which induces conjugation the lower the death-rate. If conjugation results in better adaptation to the environment in which it occurs in accord with the contention of Calkins then this is just what would be expected. My observations were, however, not extensive enough to settle the question. I refer to them here merely to indicate that the problem is subject to experimental analysis with the hope of encouraging someone to undertake its solution.

THE EFFECT OF CONJUGATION ON VARIATION IN THE RATE OF FISSION

All the essential data obtained in regard to the relation between conjugation and variation in the rate of fission are brought together in table 3. This table shows the interrelationship of the different groups of lines; the number of lines in each group the coefficient of variation to three decimal places and the dates of the different periods. The standard deviations were also calculated for all of the periods. They were, however, in such close agreement with corresponding coefficients of variation that their reproduction in the table would have been superfluous.

By comparing the coefficients of variation for the ex-conjugants with the corresponding coefficients for the non-conjugants and conjugants separated, it will be seen that while in some instances the variation is greater in the ex-conjugants than in the

TABLE 3

The effect of conjugation on variation in the rate of fission in Didinium

Each of the large numbers in the columns is the coefficient of variation for the fission-rate in the different lines of a given group of lines. They represent the variation in the total number of fissions produced by the different lines in the groups during the periods indicated above. The small numbers directly below each coefficient represents the number of lines involved in the calculation of the coefficient. The brackets and arrows show the origin and the ancestry of the different groups of lines. *Conj.*, *Conj. sep.*, etc., signify the same as in Table 1.

others, e.g. (1910, 5/24–6/2), (1911, 5/2–11), (1911, 6/28–7/7) the opposite is true in others, e.g. (1911, 5/22–31), (1911, 6/18–27).

Numerous other similar instances appear throughout the whole table. I would call attention in particular to the results obtained in 1912, 6/6–7/30. In this whole series there is very little difference between the coefficients of variation in the ex-conjugants and the conjugants separated. In the first three periods it is a trifle larger in the former, in the next three a trifle larger in the latter, etc. Five lines were maintained in both groups throughout and there were very few deaths in either. This is, consequently, a very good test case. Taken as a whole the table shows conclusively that ex-conjugants in Didinium are certainly not consistently more variable in respect to the rate of fission than are the non-conjugants.

The evidence presented in the three tables seems to show clearly that conjugation in Didinium has no appreciable effect on the rate of fission or on variation in the rate of fission, and that if it has any effect on death-rate it is a retarding effect. In these respects there is a marked divergence between our results and those obtained by Jennings on Paramecium. It seems quite remarkable that in two closely related organisms the effect of an apparently fundamental process should differ so greatly.

The results taken as a whole seem to show conclusively that neither conjugation nor encystment are rejuvenating processes, at any rate, not in the sense in which Calkins has used the term: namely, to indicate a nuclear reorganization in which accumulated waste materials are eliminated. And they are clearly not in accord with the results obtained by Jennings in experiments on Paramecium. They do not, however, overthrow his theory of the functions of conjugation.

Jennings assumes that the production of favorable characters is due, in the process of conjugation, to the union of nuclear substances which differ in potency. If therefore, the nuclear potency of the conjugants is the same, one would not, in accord with Jennings' contention, expect any favorable effect. The didinia used in the experiments described in the preceding pages were very closely related. It may, consequently, be maintained that the failure to obtain any effect by conjugation was due to the similarity of the nuclear potency of the conjugants. If this is true it is obvious that our results do not militate against the contentions of Jennings as set forth above.

SUMMARY

1. Various groups of pure lines of didinia, all but the first two originating from a single individual, were propagated in parallel series and studied, with certain intermissions, from April, 1910, till May, 1914. During this time there were produced without conjugation in one of the groups an average of 1646 generations per line and without encystment an average of 1035 generations per line. At the close the stock was very weak but it did not die out. It is, therefore, not probable that either of these processes is necessary for continued existence in Didinium.

2. From time to time throughout the entire experiment new groups of lines were started from old ones, some after conjugation, others after encystment and still others without either conjugation or encystment. There were, consequently, continuously present a number of groups of lines which differed in the number of generations produced since conjugation or encystment had occurred. In some instances this difference was very great.

3. The rate of fission varied greatly throughout the experiment, owing largely to changes in temperature but at any given time it and also the death-rate were practically the same for all of the groups of lines regardless of the distance removed from conjugation or encystment. There was, therefore, no evidence indicating the presence of cycles related to these processes.

4. There was no evidence obtained indicating that conjugation or encystment has any appreciable effect on death-rate, fission-rate or variation in fission-rate. This would indicate that neither of these processes is a rejuvenating process, at least not in the sense in which Calkins has used the term.

5. In one of the groups of lines, 721 generations after conjugation and 197 generations after encystment, some of the offspring suddenly began to divide more rapidly than others. The difference in the rate of fission in these two sets of individuals

remained fairly constant throughout the remainder of the experiment, 315 days. During this time one set produced an average of 838 generations per line, $2\frac{2}{3}$ per day, the other an average of 634 generations per line, 2 per day.

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