SOME ASPECTS OF CONJUGATION IN *STENTOR COERULEUS*

by

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ABSTRACT

Conjugation in Stentor coeruleus was investigated using two experimental methods which regularly yielded large numbers of mating pairs. One method involves a particular culture technique, the other requires a mixture of cells from different stocks. Mating pairs appeared in the form of bursts of conjugation, either induced by mixing certain stocks or occurring spontaneously in some stock cultures. Spontaneous bursts, in the majority of cases, occurred during a definite interval in the development of a culture. Morphologically distinct pre-conjugator cells appear immediately before as well as during the initial stages of a burst of conjugation. Mating pairs were formed by the union of two pre-conjugators.

Mixing eight stocks in all possible combinations of twos and observing their subsequent response revealed they were separable into two complementary mating types. The majority of mating pairs formed in mixtures of stocks consisted of individuals of different mating types.

Evidence is presented which is compatible with the hypothesis that cell to cell contacts between individuals of differing mating type are necessary for the initiation of a mating reaction.

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INTRODUCTION

An essential characteristic of a diploid organism is its ability to effect, in one way or another, the recombination of its genes in its offspring. Among the ciliated protozoans this is brought about by the sexual process of conjugation which involves the temporary union of two individuals, during which they exchange parts of the nuclear apparatus.

The phenomenon of conjugation has been observed and studied in a large number of ciliated protozoans. However, information concerning sexual reproduction in Stentor is lacking. Conjugation in Stentor coeruleus has been observed by Moxon (1869), Balbiani (1891), Hamburger (1908), Mulsow (1913), and more recently by Tartar (1961), Burchill (1967), and Harden and Holland (1968). The early workers found that exconjugants did not survive long after engaging in sexual reproduction. Mulsow, however, was able to keep them alive long enough to examine the cytological events of conjugation. His studies showed that the events of conjugation accomplish the recombination of chromosomes in a manner similar to that found in other ciliates; namely, the fusion of haploid pronuclei and subsequent renewal of the macronucleus from micronuclear derivatives. Tartar in reviewing the subject suggests that the proper conditions for conjugation seldom occur. He draws attention to studies in which Stentor cultures have

been carried on continuously for several years with very few or no mating pairs detected.

Recently, two separate studies (Burchill, 1967; Harden and Holland, 1968) reported the occurrence of abundant conjugation in mass cultures of Stentor coeruleus. Burchill found that conjugation occurred most often in cultures which had been fed recently, observing up to fifty percent of population involved in conjugation at one time. Harden and Holland (1968) discuss the effects of conjugation on a large number of conjugating pairs, stating that S. coeruleus seems to be able to follow one episode of conjugation with another after a short interval. They also observed a high mortality rate among exconjugants but were unable to demonstrate any increase in mortality rate due to inbreeding.

To date, all studies of sexual processes in Stentor have been confined to the occurrence of conjugation in mass collections or cultures. Such studies, although providing a considerable amount of knowledge, are limited because the occurrence of conjugation is rather unpredictable. Recent attempts to induce conjugation (Tartar, 1961; Burchill, 1967) in this genus have been unsuccessful.

In several ciliate genera conjugation has been brought under stringent experimental control by virtue of the fact that mating type differentiations account for the union of cells. When cultures of complementary mating type are mixed a mating reaction occurs because of cross-mating between

the two types. Until the particular mating system of a species is known the controlled matings required for genetic studies are impractical.

The purpose of this study was to investigate sexual reproduction in Stentor coeruleus with the aim of developing an experimental system with which to control conjugation, and to add to the general knowledge of conjugation in this genus. The study was occasioned by the unexpected discovery of persistent conjugation in stock cultures of this ciliate.

METHODS AND MATERIALS

1. Stocks of Stentor

The study was conducted using nine different stocks of Stentor coeruleus which were obtained from various sources (see Appendix). They were designated as follows: C, DF, S, SC, ST, T, CH, H and W. To provide genetic uniformity, each stock was established in culture from the vegetative progeny of a single cell (thus representing a clone) and thereafter was maintained separately in continuous culture. Five of the stocks (C, DF, S, SC, T) were genetically distinct because each one was derived from a different natural population. The collection sites range from the west coast to the east coast of North America; therefore these stocks should represent a wide range of genetic diversity. Stocks CH and H were originally derived from stock T and had been maintained in separate laboratories for a period of at least two years before being established in culture for the present study. Stock ST was originally derived from organisms belonging to stock SC but was also maintained in a separate laboratory before being established in culture for the present study. The origin of stock W is not known.

2. Petri Dish Culture Technique

After being established in culture each stock was maintained in continuous culture by the following subculture

method.

All cultures were maintained in sterile plastic petri dishes (100 x 25 mm). Each petri dish was coated on the bottom with a thin layer of one per cent agar (De Terra, 1966). The agar layer appeared to facilitate the attachment of stentors to the bottom of the petri dish; in its presence consistently good cultures were produced. The culture dishes were prepared by addition of approximately 75 ml of millipore filtered pond water along with two boiled wheat grains. To initiate a culture an inoculum of 0.5-1.0 ml of water, containing a species of colorless flagellates (cultured separately in the same medium), was added to the culture dish followed 24 hours later by the introduction of 25-50 stentors. The colorless flagellates, closely resembling Rhabdomonas costata, acted as food organisms. All cultures were maintained in the laboratory at room temperature (21^{o}-25^{o}C). Stocks cultured in this manner consistently yielded a vigorous population of rapidly dividing stentors which approached maximum density in 12-16 days. After reaching maximum density the population entered a stationary phase of growth during which few, if any, dividing stentors were present. Subculturing was carried out when the stentors were well into the stationary growth phase but before the culture began to decline (the culture was then 20-25 days old). All cultures were maintained for 30 days regardless of their time of subculture. Cultures were observed daily or every second day

to follow their progress from the time of initiation to the 30^{th} day in culture. Observations were made by transmitted light using a Wild M-5 stereomicroscope with substage illumination.

3. Photography

Organisms were photographed, as they were, in the culture dishes in order to record the events of conjugation in an undisturbed state. Photomicrographs were taken with Kodak high contrast copy film using a Wild Mka 1 camera which was mounted directly on a Wild M5 stereomicroscope. The light source for photography was provided by a Leitz micro-flash device with a flash duration of 1/1000 second.

4. Induction of Conjugation

A method was developed which successfully induced conjugation in Stentor. The essential feature which is necessary for the induction of conjugation is the mixing of sexually reactive cells from two different stocks. This feature was incorporated into several experiments designed to demonstrate the extent of mating between all stocks, the extent of cross-mating between cells of two stocks, and the actual stimulus which leads to the formation of mating pairs. Discussion of each of these experiments will be reserved for later sections to avoid any confusion which might arise from an awkward separation of a discussion of the methods used and the results obtained.

RESULTS

The initial observations in this investigation were made with the unexpected discovery of conjugating pairs in several cultures of stock S stentors. At that time it was considered possible that the culture technique was providing conditions which favored conjugation. With this in mind a number of subcultures were made using exactly the same technique as before; again, numerous conjugating pairs appeared. These preliminary observations lead to the start of an investigation which ran from November 1966 to May 1968, the results of which are presented in this thesis.

1. The Appearance of Conjugation in Stock Cultures

Conjugation, often involving large numbers of cells, was observed frequently throughout the period of study in stock cultures maintained in the manner previously described. Cultures of each of nine stocks maintained in continuous subculture were observed daily or every second day to follow their progress from the time of initiation to the 30^{th} day in culture. After 30 days the cultures were declining and data were no longer recorded.

A total of 602 stock cultures were made and observed; of these, 292 (49%) contained mating pairs at some time during their development. The number of pairs present at one time in a culture dish varied from less than 1% to greater than 30% of the total population. These figures are derived from

the accumulated data for all nine stocks and, as such, are somewhat misleading. They are included here merely to indicate that the culture method does appear to provide conditions which are favorable for sexual reproduction. A more detailed inspection of the data obtained from the consideration of each stock individually, revealed a definite variation between stocks in their tendency to produce cultures which contained mating pairs (Table I). These data show a well defined tendency for some stocks (CH, DF, H, S, T and W) to produce subcultures which regularly contain mating pairs, but at the same time show other stocks (C, SC and ST) to have little or no tendency to produce cultures which contain mating pairs. On this basis, the stocks can be divided into two groups: those which regularly conjugate within stock cultures, and those which do not. This division is further substantiated by the observation that conjugation in cultures of stocks C, SC and ST never involved the formation of more than five mating pairs in any culture, while, in contrast, conjugation in cultures of stocks CH, DF, H, S, T and W usually involved the formation of large numbers of pairs, often numbering in the hundreds.

Petri dish cultures initiated with food organisms and stentors usually exhibited a definite sequence of events with respect to their growth pattern and the appearance of mating pairs through the 30 day observational period. During the first few days the food organisms multiplied rapidly followed closely by an increase in the number of

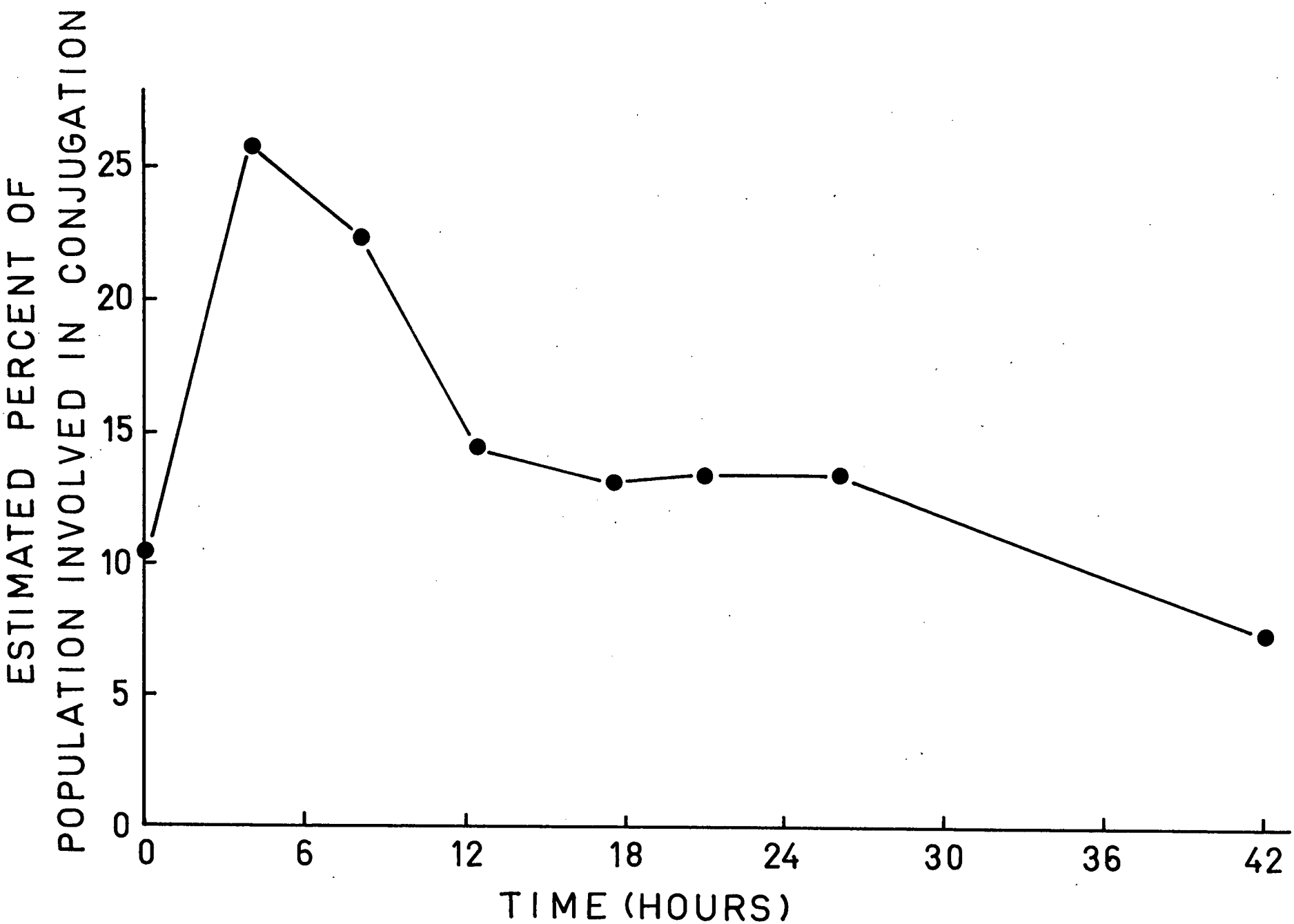
Table I. The appearance of mating pairs in stock cultures.

stocks	total number of subcultures made	number of subcultures containing mating pairs
C	51	6(12%)
CH	40	19(48%)
DF	11	8(73%)
H	123	85(64%)
S	242	104(43%)
SC	19	1(5%)
ST	19	0
T	47	44(94%)
W	50	25(50%)

stentors. The population of stentors approached maximum density (estimated from the decreasing number of dividing individuals) after about 14-16 days. During the following five or six days the population entered a stationary phase during which few dividing stentors appeared. As mentioned previously, by the time a culture was 30 days old it usually had begun to decline. When mating pairs appeared in a culture dish, they most frequently did so in the form of a burst of conjugation lasting several days. In any conjugation burst a large number of mating pairs appeared on the first day, however, pairs in decreasing numbers were formed on succeeding days. The graph in figure 1 follows the change in the percentage of the population involved in conjugation through a typical burst. The data for the graph were obtained from direct counts taken at intervals during the course of the burst. The counts ranged from 730 to 1,273 cells, each cell being scored as to whether or not it was conjugating. From these figures an estimate was made of the percentage of the total population involved in conjugation at the time of the count. A significant observation from this curve is that during the four hour interval between the first and second counts there was approximately a 2.5 fold increase in the percentage of mating cells. Equally as striking is the further observation that 24 hours earlier there were no paired cells present in the culture.

When a burst of conjugation appeared in a culture, it did so only once and at a definite stage during the 30 day

Figure 1. Estimated change in the percentage of the population involved in conjugation through a spontaneous conjugation burst. (The first count was taken as time zero.)


ESTIMATED PERCENT OF POPULATION INVOLVED IN CONJUGATION
0
5
10
15
20
25
0
6
12
18
24
30
36
42
TIME (HOURS)

life of the culture. To demonstrate the stage at which conjugation appeared, a number of cultures of stocks CH, H, S, T and W were set up and observed daily to record the exact number of days elapsed from initiation to the first appearance of mating pairs. Recall that these five stocks belong to the group which regularly produced cultures containing large numbers of mating pairs. The results from the above experiment (shown in Table II) indicate the burst of conjugation begins in the interval 7-24 days after the initiation of the culture, with an overall mean of 12.2 days. A closer look at the data reveals that 98% (123 of 126 total)[1] of the culture dishes had the beginning of the conjugation burst fall in the interval 7-18 days. This adjusted figure probably represents a more realistic interval than that previously stated. Under the culture conditions of this investigation a prevalent observation emerges: mating pairs rarely, if ever, appeared in young cultures (1-6 days) or in older cultures (19-30 days), but pairs did appear with some regularity in cultures which were 7-18 days old. The reasons for the appearance of mating within this relatively distinct interval may reside at least in part with the nutritional state of culture and will be dealt with in the discussion.

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[1]The three numbers excluded are marked with an asterisk in the raw data which appear in Figure II.

Table II. Number of days from the initiation of the culture to the first appearance of mating pairs in stocks CH, H, S, T and W.

CH	H	S	T	W
16	10	18	7	10
11	7	14	11	7
11	7	14	11	7
9	7	11	12	11
16	10	10	11	7
16	12	15	11	10
13	10	14	9	10
14	15	18	10	10
16	10	11	12	10
14	24*	12	12	14
14	18	12	14	11
11	14	11	16	11
11	18	11	16	14
	17	10	10	14
	14	12	19*	
	18	9	10	
	13	9	10	
	10	10	11	
	12	16	14	
	12	12	14	
	12	10	11	
	13	12	19	
	12	8	20*	
	12	8	16	
	11	8	16	
	10	8	13	
	11	9	14	
	12	9	14	
	14		14	
	14		14	
	11		11	
	10		10	
	12		14	
	10		10	
	10		14	
	10			
MEAN:- 13.2	12.3	11.5	12.9	10.4

OVERALL MEAN:- 12.2

* These figures were excluded to give the adjusted interval (see text for explanation).

2. Pre-conjugators and the Formation of Mating Pairs

The accumulated observations through a great many bursts of conjugation, both in stock cultures and in induced bursts (induced bursts will be discussed in a later section), make it possible to describe some of the features which characterize the mating behavior of Stentor. As previously mentioned, the beginning of a burst of conjugation was characterized by the appearance of a great many mating pairs. At the same time, the early stages of a burst were marked by the appearance of numerous morphologically distinct cells which are designated as "pre-conjugator cells". These unique cells are distinct from normal vegetative stentors in having a portion of their left frontal field and associated membranellar band folded down (compare Figure 2a and b with c and d). Within the folded-down portion there appears a prominent bulge (see Figure 3) which eventually becomes the locus of the initial mating union when two cells join to form a mating pair. Preconjugators were most evident during the first day of a conjugation burst, becoming less numerous on succeeding days. The actual mating behavior which results in the joining of two preconjugators to form a mating pair involves an active series of events directed toward the formation of a mating pair.

Preconjugators usually remained attached to the bottom of the culture dish by their holdfasts thus leaving their "head" ends free to wave about. Two cells, which were about to join, first oriented themselves in such a way that the folded-down

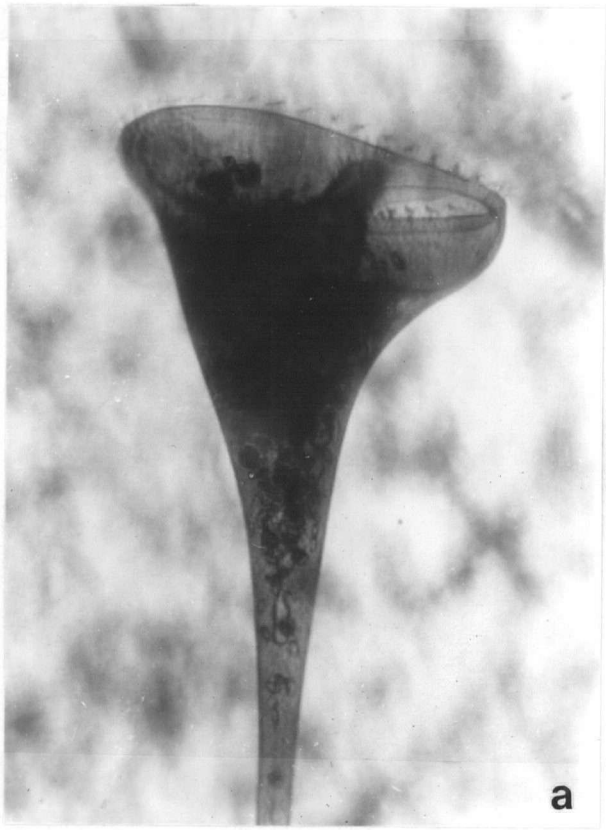
Figure 2. Representatives of normal vegetative individuals and pre-conjugators. (For the preconjugators the folded-down portion of the frontal field is indicated by a bracket.) Magnification 110x.

a) Ventral view of a normal cell.

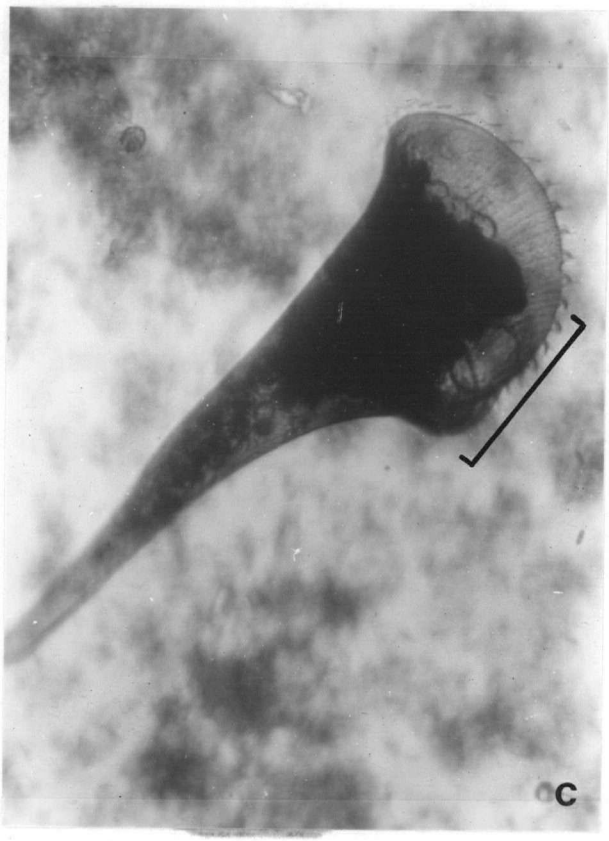
b) Anterior view of a normal cell.

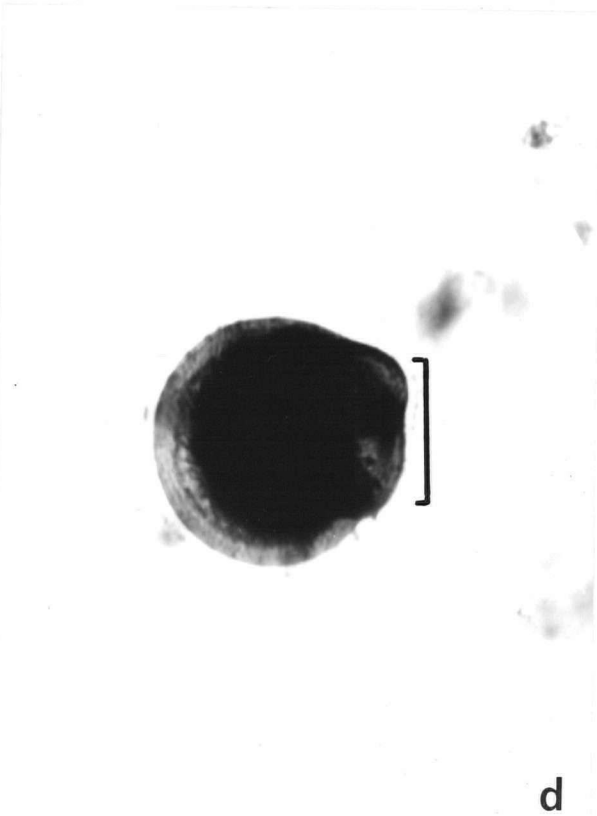
c) Ventral view of a pre-conjugator.

d) Anterior view of a pre-conjugator.


a


b


c

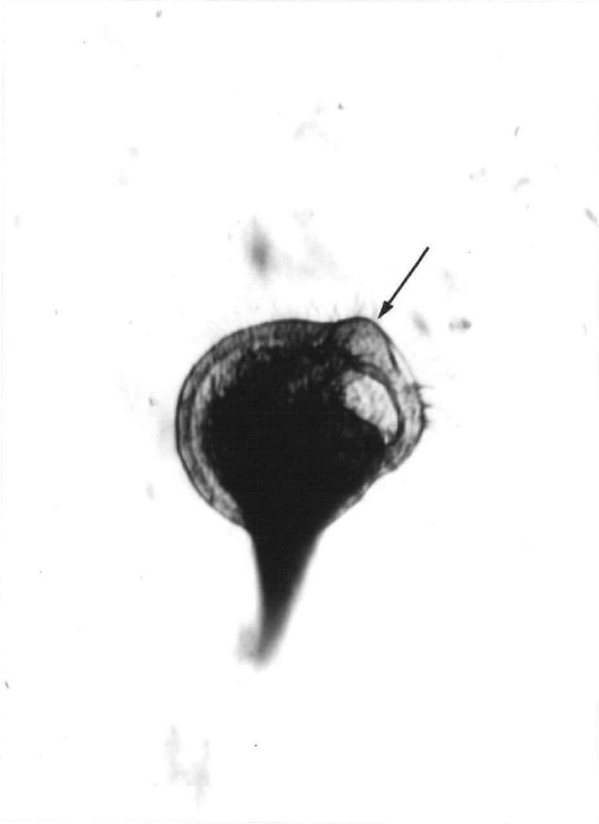

d

portions of their respective frontal fields were in close apposition (Figure 4). Often they remained in this position for up to fifteen minutes with the cilia of their membranellar bands touching and continually beating. Eventually the bulges on each cell touched and stuck, thus uniting them (Figure 5). At this point the union between the cells was not very strong for if they were stimulated to contract (Figure 6) they often pulled apart. The folded-down portion of the frontal field was never seen to stick to any other area of any other cell, although having ample opportunity to do so as it made contacts with neighbouring cells. Therefore, the complementary stickiness appears to reside only within the folded-down portion of the frontal field and associated membranellar band, possibly only on the bulge itself. The formation of a mating pair was always the result of two pre-conjugators adhering. On no occasion were two vegetative cells or a vegetative and a pre-conjugator seen to join together.

After two cells became firmly united they usually remained relatively motionless as the union between them broadened. They remained joined for varying lengths of time, usually longer than 24 hours. Figure 7 (a, b, c, d, e) shows a sequence of photomicrographs of a mating pair, tracing its development from initial adhesion till just before separation into two exconjugant cells, a total of 28 hours.

Figure 3. Anterior view of a pre-conjugator showing the bulge (indicated by the arrow) within the folded-down portion of the frontal field. Magnification 110x.

Figure 4. Two pre-conjugators with the folded-down portion of their frontal fields in close apposition; bulges not yet joined. Magnification 110x.



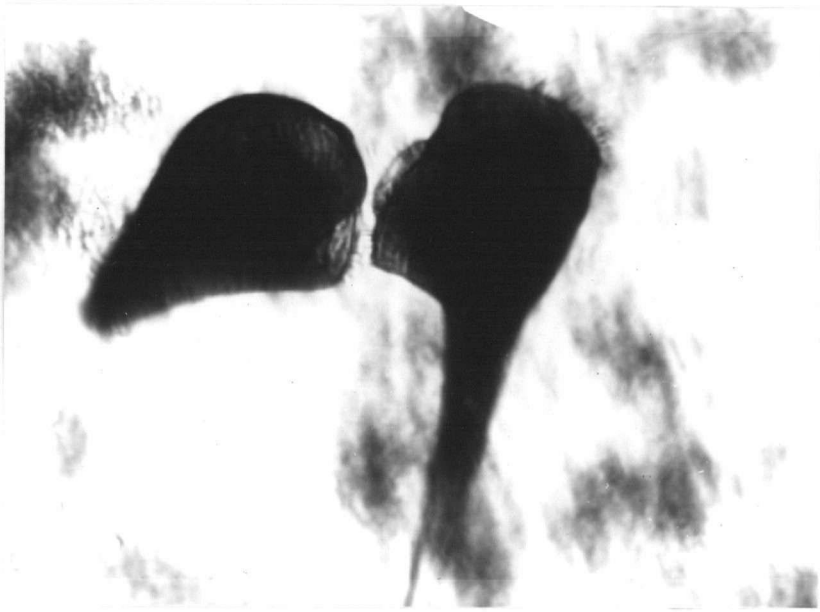


Figure 5. Two pre-conjugators with bulges just joined. Magnification 110x.

Figure 6. Contracted cells just before they pull apart. Magnification 110x.

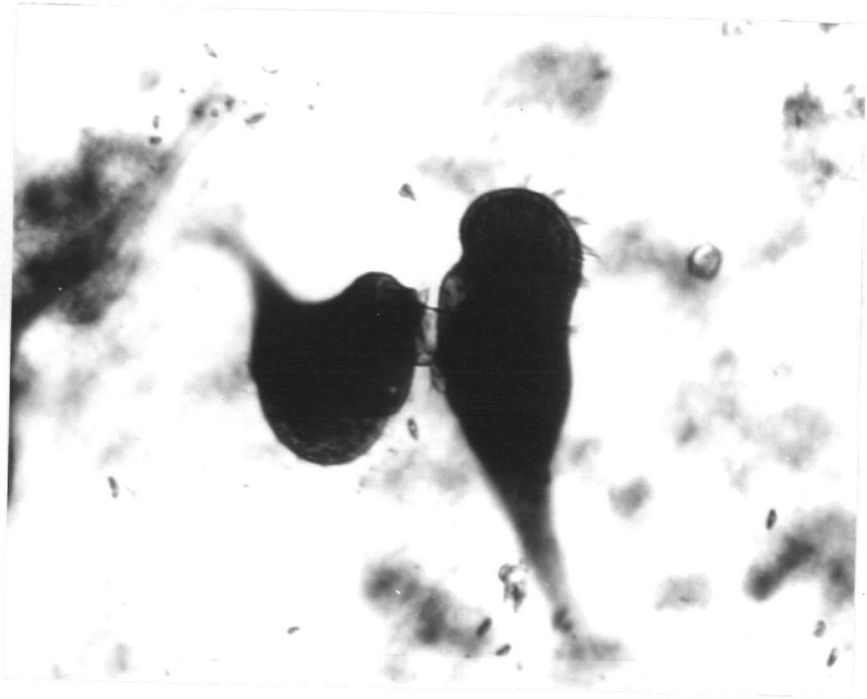


Figure 7. A sequence showing the development of a mating pair from initial adhesion till just before separation, a total of 28 hours. Magnification 74x.

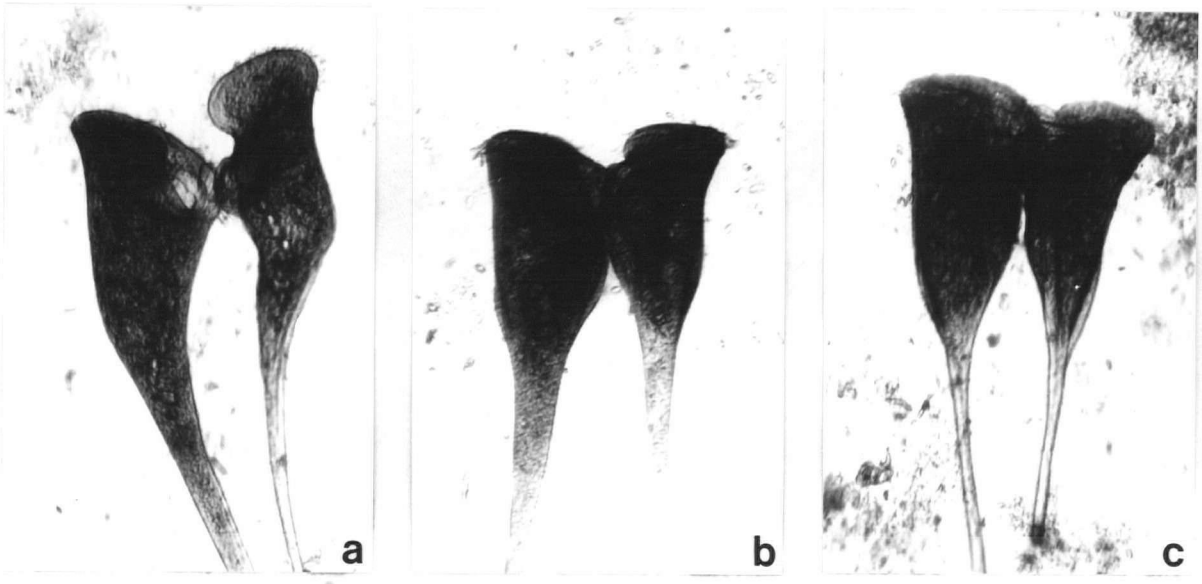
a) Bulges just united.

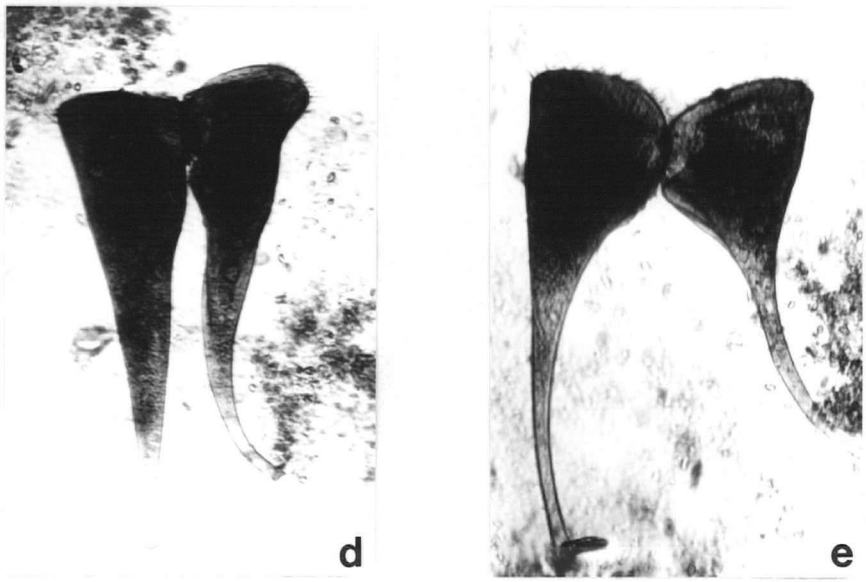
b) 4 hours.

c) 9 hours.

d) 21 hours.

e) 28 hours, separation came during the following hour.


a
b
c


d
e

3. Production of Selfing Clones

The term selfing, as used by students of ciliate genetics, refers in its broadest sense, to the occurrence of conjugation among individuals derived, by asexual reproduction, from a single parent cell (i.e. intraclonal conjugation). In this respect Stentor appears to have a high tendency for selfing since six of the nine stocks regularly produced subcultures in which intraclonal conjugation occurred. To further investigate the selfing ability of Stentor, single cells were isolated, placed in separate petri dish cultures and allowed to reproduce asexually. In each case, the resulting progeny were observed for the presence of intraclonal conjugation. Single cells from two sources were selected: normal vegetative stentors from each of the nine stocks and stentors from stock S which had just become paired at conjugation. For the former group a single vegetative cell was isolated from each stock and placed separately into a petri dish culture. The source of cells for the latter group requires explanation in some detail. In order to obtain stock S cells which had just become paired at conjugation it was necessary to closely observe a culture in the early stages of a conjugation burst in which there were a large number of preconjugators evident. When two pre-conjugators were seen to come together and stick firmly they were quickly removed and transferred along with approximately 2 ml culture fluid into a small petri dish (35 x 10 mm). Before any nuclear exchange could take place, the connection between the cells

was severed with a fine glass needle. Each of the now separated cells was placed into a separate petri dish culture. A total of 12 cells (6 split pairs) was prepared and grown in culture by this procedure.

The progress of all 21 cultures produced by the two above sources was followed for 30 days. If, in any of the cultures, no conjugation was observed, then those dishes were subcultured and observed for an additional 30 days. This process of subculturing was repeated with each culture dish until conjugation appeared, or until five subcultures had been made. The results (Table III) show that single cells from stocks CH, DF, H, S, T and W possess the ability to produce clones which will exhibit intraclonal conjugation. Further, the results show that cells isolated just after they have become paired at conjugation also have the ability to self.

4. Induced Conjugation

In some cases, conjugation was found to be induced by the introduction of cells belonging to one stock into a culture dish containing cells of different stock. The essential feature, necessary for the induction, is to mix sexually reactive cells from two different stocks. Mixtures were accomplished by the following technique. Two 12 day old petri dish cultures, not yet selfing, belonging to different stocks were selected. One culture, which was to act as the donor, was placed on the stage of the stereomicroscope and the other

Table III. Intraclonal conjugation (selfing) in cultures started from single cells

Source of single cells*		Number of subcultures made after the initial culture	Presence of mating pairs in the final subculture
Stock C		5	no
Stock DF		0	yes
Stock S		1	yes
Stock SC		5	no
Stock ST		5	no
Stock T		3	yes
Stock CH		1	yes
Stock H		0	yes
Stock W		3	yes
Split pair:	cell a	1	yes
	cell b	died	___
Split pair:	cell a	2	yes
	cell b	0	yes
Split pair:	cell a	died	___
	cell b	2	yes
Split pair:	cell a	1	yes
	cell b	1	yes
Split pair:	cell a	3	yes
	cell b	1	yes
Split pair:	cell a	1	yes
	cell b	1	yes

* All split pairs were from stock S.

beside the microscope. With the aid of the microscope and using a Pasteur pipet, approximately 300 stentors were carefully removed from the agar surface of the donor culture dish and introduced into the other culture dish. Subsequent observation of the recipient culture dish revealed a striking sequence of events which will be referred to as the mating reaction. After a refractory period lasting about 5-7 hours there was the gradual appearance of numerous pre-conjugator cells. This was followed immediately by the formation of mating pairs. The reciprocal experiment, in which the donor stock culture of the previous experiment became the recipient of cells from the extraneous stock, showed a similar mating reaction, again leading to the formation of mating pairs. The induced conjugation bursts were different in some respects from the bursts of conjugation which occurred spontaneously in petri dish cultures of the nine stocks. First, there were far more pre-conjugators found in the induced conjugation bursts. Pre-conjugators numbering in excess of 100 were frequently seen at one time during induced conjugation bursts, whereas, in spontaneous bursts usually less than forty pre-conjugators were evident at any one time. Secondly, it was possible, in the induced bursts, to observe many mating pairs being formed as their bulges adhered, while in spontaneous bursts it was difficult to find mating pairs just as they were being formed. This difference is probably due to the greater number of pre-conjugators being present in the induced conjugation burst.

5. Specific Mating Between Stocks

Early in the investigation it was found that not all combinations of cells from the various stocks gave the induced mating reaction. In order to determine which combinations would result in an induced conjugation burst, the stocks were mixed together in all possible combinations of twos[2]. To accomplish this, each stock was tested individually by adding cells from the remaining seven different stocks (donors) to a series of petri dish cultures (recipients) of the stock being tested. The control in each case was made by adding cells which belonged to the stock being tested, to a culture of that same stock. The mixtures were conducted in the manner previously described for the induction of conjugation. Each mixture was subsequently observed for the appearance of an induced conjugation burst. A mixture was assumed to be lacking in any capacity to give a mating reaction if after 24 hours no pre-conjugators or mating pairs were formed. The results (Table IV) show the stocks to be divisible into two groups with respect to their mating reactivity in mixtures: group I consists of stocks C, SC and ST, while group II consists of stocks S, T, CH, H and W. No mating reaction was observed in mixtures of cells of two stocks belonging to the same group. But, a mating reaction did occur in all mixtures where cells from any stock in group I were combined

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[2]Stock DF was not included in this experiment because it was not obtained until the latter part of the investigation.

Table IV. Specific mating in mixtures of stocks.

Donor stocks	Stocks tested (recipients)*							
	C	SC	ST	S	T	CH	H	W
C	-	-	-	+	+	+	+	+
SC	-	-	-	+	+	+	+	+
ST	-	-	-	+	+	+	+	+
S	+	+	+	-	-	-	-	-
T	+	+	+	-	-	-	-	-
CH	+	+	+	-	-	-	-	-
H	+	+	+	-	-	-	-	-
W	+	+	+	-	-	-	-	-

* Each vertical column represents a series of cultures of a single stock which were tested for their ability to give a mating reaction with cells from the donor stocks. A plus (+) sign indicates a mating reaction occurred. A minus (-) sign indicates no mating reaction occurred.

with cells from any stock in group II.

These results strongly suggest that the formation of mating pairs in appropriate mixtures is the direct result of the presence of two complementary mating types. It, therefore, seems reasonable to tentatively assign mating types to group I and II. Hence, the stocks in group I become mating type I and those in group II become mating type II.

6. Demonstration of Cross-mating

It was desired to determine whether the mating observed during induced conjugation bursts was in the form of cross-mating, with one cell of a pair belonging to one stock and the other cell belonging to the other stock.

Several preliminary observations led to the early belief that cross-mating did occur during induced conjugation bursts. These came from the scrutiny of individual mating pairs formed in mixtures; in the majority of pairs, the two cells were recognizably different with respect to size and intensity of pigmentation, relative to each other. Figure 8 shows a characteristic mating pair formed in a mixture of stock C and stock S cells. The larger more darkly pigmented cell almost certainly belongs to stock S, whereas the smaller, more lightly pigmented cell almost certainly belongs to stock C. This judgement was reinforced by measuring the diameter of contracted cells of both stocks; measurement revealed a difference in size such that cells of stock S averaged 272 microns while cells of stock C were

smaller, averaging only 220 microns. Further, when cells from stocks S and C were placed together in a drop of water on a slide and observed with the stereomicroscope, the difference in pigmentation intensity, although not great, was apparent.

Labelling with carmine was used to investigate the occurrence of cross mating more fully and to obtain quantifiable results. Labelled cells of a group I stock were mixed with unlabelled cells of a group II stock and both cells of the resulting mating pairs were individually scored on the basis of three distinguishing characteristics: presence or absence of label, light or dark pigmentation with respect to each other, and large or small size with respect to each other. For the experiment, stocks C and S were chosen because the difference between them, with respect to size and pigmentation,was most easily seen.

Labelling was accomplished by the introduction of a carmine particle suspension (prepared by suspending 0.1 g powdered carmine in 1 ml culture fluid) into a culture containing stock C. Immediately, the cells began to ingest the carmine and after 15 minutes their cytoplasms contained large quantities of the label. Following this treatment, approximately 300 newly labelled cells were removed, washed by centrifugation in three changes of fresh medium to remove any free carmine particles, and then added to a petri dish culture containing stock S. Twelve hours later the resulting mating pairs were removed, placed one at a time on a

glass slide in a drop of culture fluid, and separated with a fine glass needle. Each cell of the pair was then placed in a separate drop of culture fluid on the slide, squashed lightly with a cover glass (Figure 9), and examined for the presence of carmine particles using the steromicroscope. Altogether, three separate experiments were performed with stocks C and S using the above labelling technique. The results (Table V) for each mating pair were scored on the basis of pigmentation, size and presence of carmine, resulting in the emergence of six classes of mating pairs from the combined results of all three experiments. However, a close examination of the data in Table V revealed that some classes may be amalgamated to give a total of three distinct classes: first, a cross-mating class (combining classes 1, 2 and 3) in which mating pairs consist of one stock C cell and one stock S cell; secondly, a selfing class (combining classes 4 and 5) in which mating pairs consist of two stock C cells; and thirdly, an additional selfing class (class 6 only) in which mating pairs consist of two stock S cells. This redistribution is based on the probability that some of the labelled stock C cells had extruded the foreign carmine particles thus leaving them unlabelled. This assumption is supported by the presence, after 12 hours, of carmine particles on the bottom of the culture dish where at the beginning of the experiment there had been none.

Figure 8. Mating pair from a mixture of stocks C and S. Magnification 104x.

Figure 9. Squashed pair from a mixture of labelled stock S cells and unlabelled stock C cells. (The arrows indicate the carmine particles.) Magnification 104x.

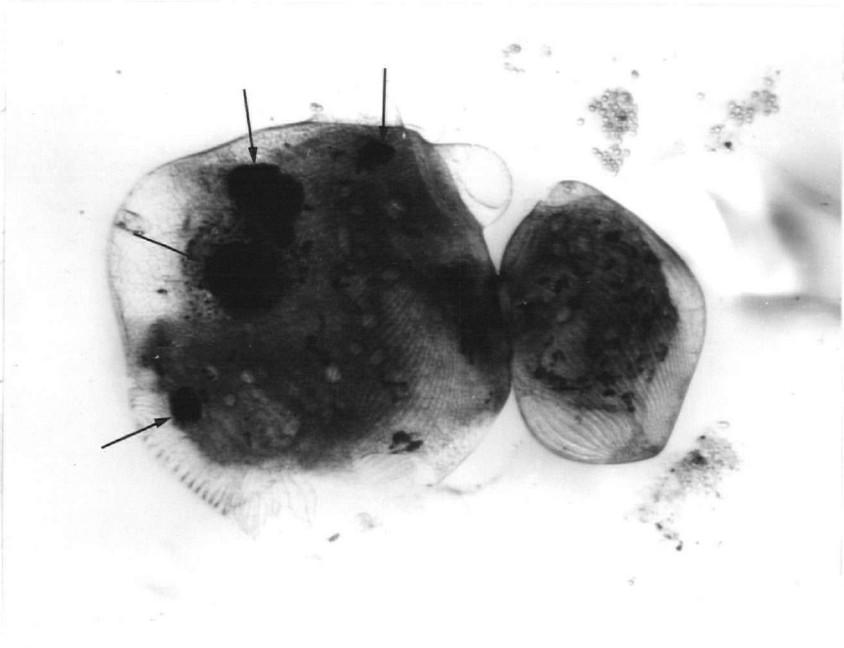


Table V. Observed Classes of Mating Pairs Scored on the Basis of Relative Pigmentation, Presence of Carmine, and Relative Size.

	Split pair									
	Cell a			Cell b			Number of mating pairs			
class of pair	pigment	carmine	size	pigment	carmine	size	Expt 1	Expt 2	Expt 3	Total each class
1	lgt	+	sml	drk	-	lrg	33	29	41	103
2	lgt	-*	sml	drk	+	lrg	4	2	4	10
3	lgt	+	sml	drk	+**	lrg	0	1	0	1
4	lgt	+	sml	lgt	+	sml	1	1	1	3
5	lgt	+	sml	lgt	-*	sml	0	0	1	1
6	drk	-	lrg	drk	-	lrg	1	0	1	2
						Totals:	39	33	48	
									Total pairs sampled:	120

Symbols and abbreviations: + carmine particles present; - no carmine particles present; lgt = lightly pigmented cell; drk = darkly pigmented cell; sml = smaller cell; lrg = larger cell.

* The absence of carmine particles in these cells is probably due to the cells having extruded the foreign particles (see text for a further explanation).

** This cell contained a single very small carmine particle which was probably picked up from the bottom of the culture dish.

The redistributed results strongly suggest that 95% of the mating pairs formed in the mixture were of the cross-mating type, while the remaining 5% were of the selfing type.

The above labelling experiments were performed only with mixtures of cells from stocks C and S. However, it is significant that in mixtures of other stocks, which resulted in an induced conjugation burst, the majority of mating pairs formed appeared to be of the cross-mating type, judged on the observed differences in size and pigmentation of the two cells making up the pairs.

The high incidence of cross-mating overwhelmingly supports the conclusion that the mating reaction, which occurred only in certain mixtures, was due to the presence of complementary mating types (i.e. mating types I and II).

7. The Role of Cell to Cell Contacts in the Induction of Conjugation

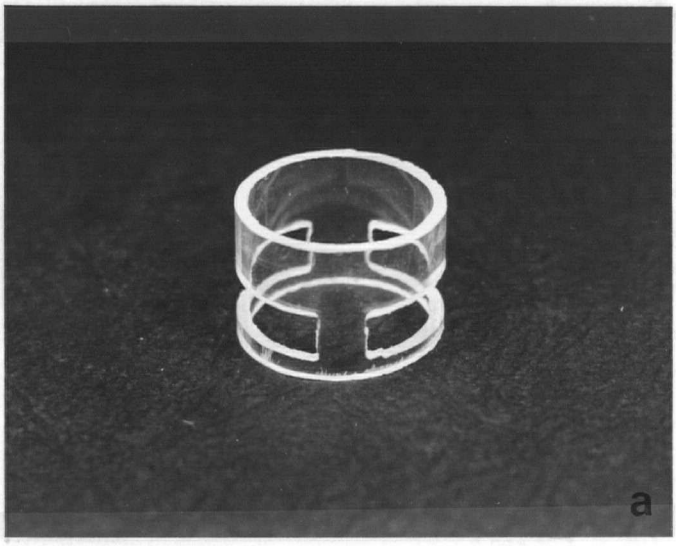
The actual stimulus which caused the mating reaction when cells from two different stocks were mixed was not obvious, but, two distinct possibilities were considered. Firstly, the cells of either or both stocks may have put some factor into the medium which caused other cells to become pre-conjugators thus leading to the observed mating reaction. The second possibility (suggested by Sonneborn in a personal communication) is that the mating reaction may have been a consequence of a "contact interaction" between sexually reactive cells. Such contact phenomena

have been found to exist in Euplotes (Heckman and Siegel, 1964) where it has been shown that the cells in a mixture of two mating types actually have to make contacts, not contacts which result in sticking together, but simply make and break contacts which stimulate the cells to become ready for conjugation some hours later.

In order to determine the cause of the mating reaction in Stentor, an experiment was performed in which a series of three diffusion chambers was used to isolate groups of cells. The central idea was to prevent the isolated cells from contacting cells of another mating type, while, at the same time, subjecting them to the influence of any diffusible factors which might cause a mating reaction.

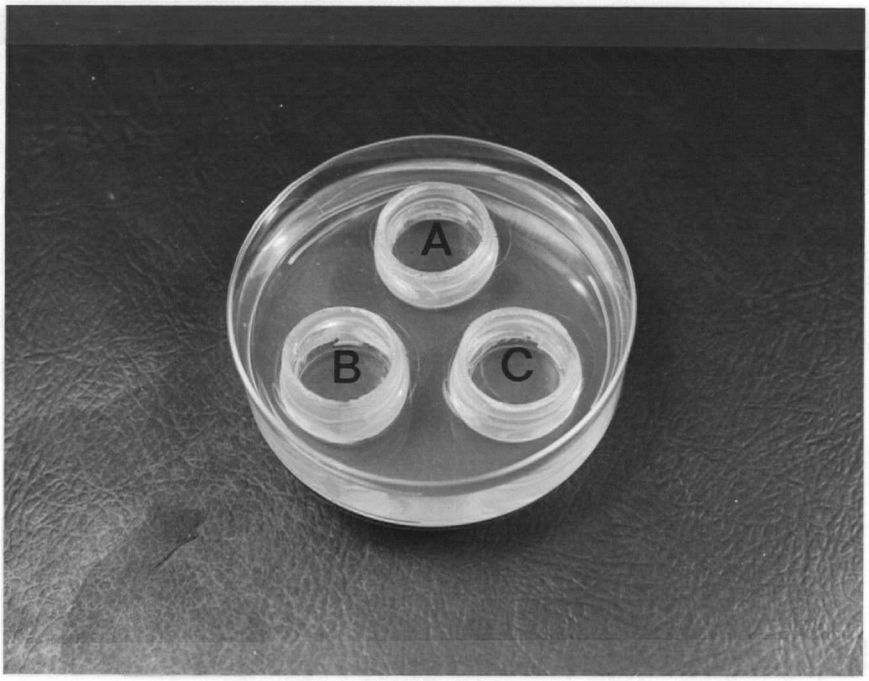
The diffusion chambers (Figure 10a, b) were made from 2 cm lengths of slotted plexiglas tubing (one inch in diameter) wrapped with nylon bolting cloth having a pore size of 62 microns (obtained from John Staniar & Co., Sherborn Street, Manchester, England). The three chambers (lettered A, B and C in Figure 11) were placed into a 12 day petri dish culture of stock C, each chamber in an area which had previously been cleared of cells. The chambers were pressed firmly into the agar layer thus effectively sealing the bottom of the chamber. Diffusion chambers prepared in this manner prevented the passage of stentors either into or out of the chamber, but did not prevent the passage of the food organisms which were about 30 microns in length.

Figure 10. Diffusion chamber (a) before wrapping with bolting cloth and (b) after wrapping is complete.


a


b

Figure 11. Petri dish culture with diffusion chambers in place.


A
B
C

To initiate an experiment, cells belonging to stocks C and S were allotted to the three chambers in the following way: chamber A received 100 stock C cells, chamber B received 100 stock S cells, and chamber C received 50 stock C cells plus 50 stock S cells. Additional cells of stock S were added to the petri dish in the area outside of the diffusion chambers. The procedure described so far represented one half of a two part experiment with both parts being executed concurrently. The other half of the experiment was the reciprocal of the part just described and was performed in the same manner except that the diffusion chambers were placed into a petri dish culture of stock S instead of stock C. Both culture dishes were subsequently observed for the presence of pre-conjugators or mating pairs. After seven hours there were, in both dishes, pre-conjugators and mating pairs present in chamber C and in the area outside the diffusion chambers; both of these areas contained cells from stocks C and S mixed together. At the same time, chamber A (containing only stock C cells) and chamber B (containing only stock S cells) showed no evidence of a mating reaction with no pre-conjugators or mating pairs being formed even after 24 hours. This experiment was repeated three times with the same results.

The results of this series of experiments indicate a lack of any diffusible factor affecting the mating ability of cells. It, therefore, seems evident that the only difference between cells in chambers A or B and those in the

other areas, lies in the inability of the cells contained in chambers A and B to make contacts with cells of a different mating type. Further evidence suggesting the absence of any diffusible factor acting in the induction of conjugation was found in the inability of conditioned medium taken from actively conjugating cultures to induce conjugation when added to a non-conjugating culture from which most of the fluid had been drained. This was repeated several times using different cultures with no apparent effect of culture fluid upon cells.

These results strongly suggest that contacts between cells belonging to different mating types play an essential role in the induction of conjugation.

8. Survival of Exconjugants

Although an extensive study of exconjugants was not made, the dominant impression acquired from the follow up of exconjugants was their apparent high mortality rate. The high mortality is evident after a conjugation burst when large numbers of sluggish, poorly pigmented cells appear which live only a few days.

In several attempts to obtain living exconjugants, a total of 80 mating pairs was isolated from different cultures at various times during the course of the investigation. In each case the mating pairs were placed separately into small petri dishes containing new medium and food organisms. After a pair separated, one cell was transferred to

another small petri dish containing new medium and food organisms. Of the total of 160 exconjugants produced by the separation of the 80 pairs, only two cells, derived from different pairs isolated from a conjugation burst in stock W, survived long enough to begin division. These two surviving exconjugants produced clones which were subcultured continuously for six months, however, no mating pairs were observed in either line of descent. The remaining 158 exconjugants died within a few days after isolation.

The most successful attempts to keep exconjugants alive were with large groups of mating pairs which were isolated and placed into a petri dish culture. Four groups of mating pairs, ranging in size from 10 pairs to 60 pairs, were isolated and placed into new petri dish cultures. One group came from a conjugation burst in a stock T culture and the other three groups came from induced conjugation bursts in mixtures of stocks C and S. Although a large number of the exconjugants in these groups died, a few did survive in each dish, eventually giving rise to a population. After 30 days in culture, during which no conjugation was observed, all four dishes were subcultured. The population which descended from the stock T exconjugants showed conjugation within the first subculture. This was also true in two of the three lines of descent which originated from the mixtures of stocks C and S. The remaining line did not show any conjugation after four subcultures.

To briefly summarize, in the presence of a high mortality

rate some exconjugants did survive and divide; in some cases they gave rise to a population of cells which conjugated in the first subculture, less than 60 days after their previous mating.

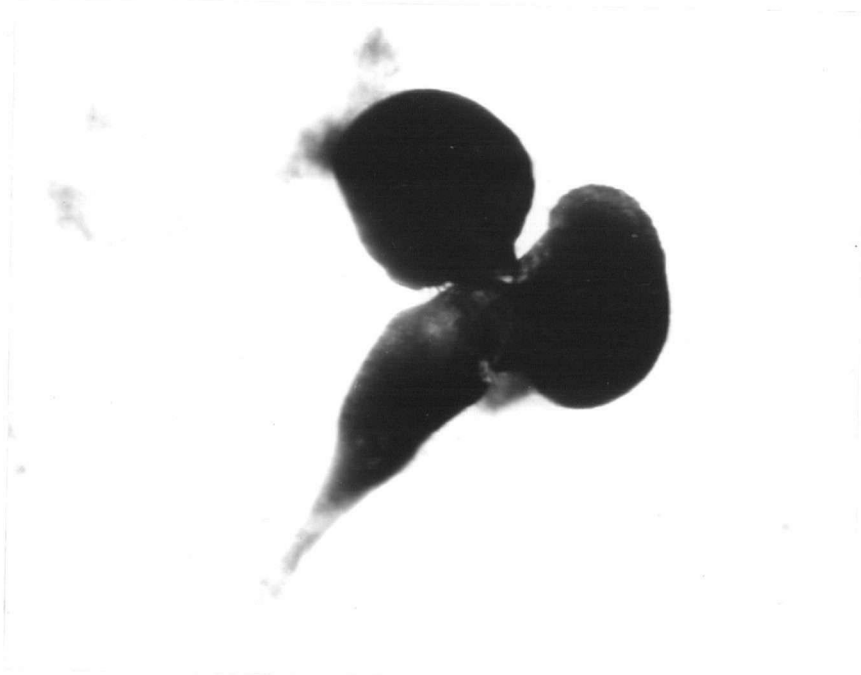
9. Abnormal Mating

During the course of the investigation, two types of abnormal mating were observed. First, a small number (approximately 15) of mating cells showing "multiconjugation" were observed in which three cells were joined together instead of the usual two. Figure 12 shows an example discovered in a conjugation burst induced by a mixture of cells from stocks C and S. A second type of abnormal mating, found in a stock S culture, was seen in a single mating pair in which one of the cells was at a late stage of division (Figure 13). The dividing cell appeared to be at stage 7 (Tartar, 1961) and remained at that stage throughout the four hour period during which it was observed.

Abnormal mating, of the two types described, appeared infrequently throughout the investigation.

Figure 12. An example of a mating triplet found in a mixture of stocks C and S. (The two larger cells appear to be stock S; the smaller cell probably belongs to stock C.) Magnification 110x.

Figure 13. Mating pair with one partner at about stage 7 of division. Magnification 110x.



DISCUSSION

In many ciliates, conjugation is most readily induced by mixing cultures of different origin. From the results of these mixtures, the cultures can be classified into two or more mating types, being classed as complementary mating types if conjugation is induced by the mixture (Sonneborn, 1957). By this definition individuals belonging to the same mating type do not conjugate (except in certain cases of selfing). But when individuals of two different mating types are mixed they will unite. Mating types have been found in seven genera of ciliates; namely, Colpidium, Euplotes, Oxytricha, Paramecium, Tetrahymena, Stylonychia, and Tokophyra (Sonneborn, 1957; Allen, 1967).

After the initial recurrent observations of conjugation in stock cultures it seemed reasonable to begin a search for mating types in Stentor coeruleus. The success of the search was enhanced by the discovery of a method to induce conjugation, in which the essential feature was the mixture of cells from two diverse stocks. It became apparent from mixture experiments that the eight stocks tested represented two complementary mating types on the basis of their response when mixed, two at a time, in all possible combinations (see Table IV). It was, therefore, possible to assign stocks to either mating type I or mating type II. This division of stocks into mating types was distinct in that mixtures consisting of cells of the same mating type did not result

in a mating reaction. But, when cells of diverse mating type were mixed, a mating reaction occurred. By this definition, stocks C, SC, and ST belong to mating type I and react in a parallel manner when mixed with stocks belonging to mating type II. In a similar way, stocks CH, H, S, T and W belong to mating type II and react in a parallel manner when mixed with mating type I stocks.

The occurrence of two mating types in the present study should not be taken to imply that only two mating types exist in this species. In several ciliate species, which have been studied extensively, rather complex systems of interbreeding mating types have been recognized. In these, a particular species may have either a single variety with two or more interbreeding mating types (e.g. Euplotes crassus Heckman, 1964) or several varieties, each with two or more interbreeding mating types (e.g. Paramecium aurelia, Sonneborn, 1957). Interbreeding between individuals of different varieties does not occur. By this interpretation, the eight stocks of the present study belong to the same variety since they constitute two interbreeding mating types. However, the small number of genetically distinct stocks (probably five; see Appendix) used in this investigation emphasizes its preliminary nature. It seems reasonable, therefore, to suppose that future studies, using larger numbers of stocks collected from widely distributed areas, will reveal additional mating types and possibly different varieties.

In most ciliates, mating pairs formed in mixtures of two complementary mating types always consist of individuals of different mating type. There have, however, been a few cases reported where a small fraction of the pairs formed in mixtures was apparently due to the union of cells of the same mating type (Hiwatashi, 1951; Metz, 1954; Katashima, 1961; Larison and Siegel, 1961). Pairs formed following mixtures of diverse cultures of Stentor regularly consist of cells of diverse types. This conclusion is based on the results of the labelling experiments in which the majority of pairs consisted of one labelled and one unlabelled cell (Table V). The explanation for the occurrence of the few pairs which appeared to be in the form of selfing is not clear. Metz (1954) showed that in Paramecium cells belonging to a single clone can mate amongst themselves after some of the individuals have made transient contacts with cells of a complementary mating type. The explanation Metz offered was that cells which have been temporarily united with individuals of a complementary mating type may in some cases acquire that mating type specificity in the course of the contact. Thus a transitory shift of mating type may occur in these cases, resulting in the formation of selfing pairs. Hiwatashi (1951) found that selfing occurred in five percent of mating pairs formed in mixtures of Paramecium caudatum.

In Stentor, selfing is also evident in the occurrence of conjugation in some stock cultures and clonal cultures.

This type of selfing probably has a different basis in its spontaneous appearance than that which appeared in mixtures. The appearance of the majority of spontaneous conjugation bursts during the interval 7-18 days after the initiation of the cultures (see Table II) seems best explained as being due to the nutritional state of the culture.

The nutritive state of the protozoan seems to be an important factor influencing conjugation in all ciliates. Sonneborn (1939) observed that in Paramecium aurelia the mating reaction does not take place in cultures that are either over-fed or completely starved. A similar situation exists in P. caudatum (Gilman, 1939) and P. bursaria and P. calkinsi (Wichterman, 1953). Geise (1939) found that food appeared to be the most important single factor in regard to conjugation in P. multimicronucleatum and that a decline in available food supply after a period of plenty was required. In practice, ciliates are brought into mating condition for conjugation studies by subjecting them to a declining nutritional regime (Sonneborn, 1957; Allen, 1967). Stentors cultured in the manner described are initially provided with a period of nutritional abundance during which they multiply rapidly. But, as they approach maximum density the food organism population dwindles and the stentors enter a period of famine. It is during the transitional period, when the food population is declining that the burst of conjugation appears. Therefore, Stentor appears to respond to a decline

in food supply after a period of plenty by selfing.

The fact that Stentor selfs brings up a problem in regard to its mating type system. The problem lies in the explanation of the basis of pair formation in a population which has not been mixed with another mating type. Selfing in pure cultures is not uncommon in ciliates in which mating types have been found to exist. The selfing conjugations previously reported for Paramecium bursaria (Jennings, 1941) P. aurelia (Kimball, 1939a; Sonneborn, 1947), P. multimicronucleatum (Sonneborn, 1957), Tetrahymena pyriformis (Nanney and Chaughey, 1955) and Euplotes crassus (Heckman, 1964) always resulted from the differentiation of individuals of complementary mating type within the selfing culture. In these cases, the selfing pairs were formed as a result of the union of cells of complementary mating type. In the few cases in which such intraclonal mating has been studied it was explained as either due to macronuclear heterogeneity and assortment of macronuclear subunits during clonal expansion (Allen and Nanney, 1958), or as a consequence of variable gene expression (Butzel, 1955). Recent studies have shown the latter to be the most likely interpretation. Heckman (1964, 1967) found in Euplotes crassus that selfing in old heterozygotes was due to a normally recessive allele being expressed. In Paramecium aurelia, selfing, interpretable as due to changes in gene activity, has been extensively studied (Sonneborn, 1966; Taub, 1966; and Bleyman, 1967a).

Returning, now, to the influence of nutrition on conjugation,

Hiwatashi (1958) found that selfing in Paramecium caudatum was susceptible to environmental (nutritional) control. Mating type changes were influenced by varying growth conditions, high fission rate favored one type, low another (Hiwatashi, 1960). Bleyman (1967b) found that in Paramecium aurelia a period of rapid growth at 27° C followed by a depletion of food at 19° C would bring about maximal reactivity, both for selfing and mating with standard testers. Both Hiwatashi and Bleyman observed that their respective selfing stocks tended to produce cultures which consisted predominantly of one mating type, but that the cultures ranged from those which did not self to those which regularly selfed, depending upon their origin and culture conditions. This observation is reminiscent of the condition which is found in the selfing stocks of Stentor.

It is interesting that all the stocks which self regularly belong to mating type II, while those which only rarely self belong to mating type I. Gilman (1941) similarly reported that in Paramecium caudatum selfing is common in some varieties and it may occur more often in cultures of one prevailing mating type than in cultures of the other.

In view of the findings reported for other ciliates, selfing in Stentor may be due to a persistent instability of mating type expression such that transitory changes in mating type would lead to the situation where two complementary mating types were present in a culture. Further, the cultures

may be predominantly of one mating type, but a period of rapid growth followed by a depletion of food may cause some individuals to temporarily change mating type, thus permitting the formation of mating pairs. This would explain the appearance of selfing after 7-18 days in culture. Depending upon the origin of the culture the mating type changes may involve type I expressing type II or alternatively, type II expressing type I. However, the distinct possibility that future investigations may reveal additional mating types capable of interbreeding with the present types makes it necessary to consider the possibility that individuals of type I or type II may be able to express a mating type not yet discovered, a situation which would result in selfing. It was possible to determine the mating type of the selfing stocks by choosing cultures which had not begun to self, presumably containing cells expressing only one mating type.

When mating types I and II of Stentor were brought together under conditions which were favorable for conjugation, the onset of mating behavior occurred only after a waiting period of 5-7 hours. The waiting period (often called the refractory period) is apparently widespread among the ciliates for it has been reported for Euplotes (Kimball, 1939b; Katashima 1959; Heckman 1963, 1964), Stylonychia (Grell, 1951; Downs, 1952) Oxytricha (Siegel, 1956), and Tetrahymena (Elliot and Gruchy, 1952; Nanney and Caughey, 1953). In mixtures of complementary mating types in Euplotes, it has been shown that during the waiting period repeated cell contacts are

made between the two mating types, leading subsequently to formation of mating pairs (Heckman and Siegel, 1964). The hypothesis here was that the contacts bring about an exchange of information which causes the cells to become ready for conjugation some hours later. Heckman and Siegel provide support for their hypothesis by demonstrating that the waiting period can be eliminated if cells of different mating types are each permitted to make previous contact with cells of a third mating type. Thus, by pre-treating the cells they found that when the mixture was made, mating occurred immediately between the first two types.

In Stentor, cell to cell contacts appear necessary for the initiation of the mating reaction. In addition, there appears to be a lack of any diffusible factor which might affect the mating ability of cells. When cells of either mating type I or type II were placed into a diffusion chamber in such a way that they could not contact cells of the other mating type but at the same time were subjected to the environmental influences of the other mating type, they did not form pre-conjugators or mating pairs. In sharp contrast, when cells of mating type I and II were placed together into a diffusion chamber the normal waiting period was followed by the appearance of pre-conjugators and, subsequently, mating pairs. The results of these experiments provide powerful evidence in support of the hypothesis that contact between cells of different mating type provides a stimulus which causes the cells to become ready for conjugation some hours

later.

The nature of the information (stimulus) which appears to be exchanged is unknown. Metz (1954) presented an hypothesis which holds that cell unions leading to conjugation are brought about by the interaction of complementary mating type substances which he said were protein in nature. Evidence indicating that protein synthesis is necessary for the appearance and maintenance of mating ability is provided by Bleyman (1964) for Paramecium aurelia and by Cohen (1965) for P. bursaria. It has been suggested (Heckman and Siegel, 1964) that upon initial contact cells of complementary mating type cannot unite but molecular amounts of mating type substances could be exchanged. Then, the "foreign" protein could stimulate synthesis of additional mating type substances so that in due course the cellular concentrations would be sufficiently high to allow cell unions. The waiting period would, of course, be the time during which the new mating type substances were being synthesized.

Stentor seems to respond to contact with cells of complementary mating type in a manner which is at least partially similar to that suggested for Euplotes by Heckman and Siegel. But, Stentor shows an additional response in the folding down of a portion of its left frontal field as it takes on the form of a pre-conjugator. If contacts with cells of another mating type stimulates synthesis of new mating type substances in this genus, then such synthesis apparently

results in a highly localized build up of such substances. The initial union at conjugation has its locus solely within the folded-down portion of the frontal field, possibly only on the bulge produced within it. Also, synthesis is evidently not completed until the cell has taken on the form of a pre-conjugator, since contacts previous to that time do not result in cell unions.

Finally, the observed high mortality of exconjugants produced in selfing cultures may be due to inbreeding degeneration resulting from the accumulation of deleterious recessive genes in homozygous condition. Nanney (1956, 1957) found that during the course of inbreeding in strains of Tetrahymena various signs of inbreeding degeneration appeared, one of which was death at conjugation. Also, there is good evidence to indicate that the use of old stocks (clones) may result in a general non-viability among exconjugants (Siegel, 1967). The stocks employed in the present study have in some cases been cultured for periods in excess of ten years. A large portion of the age-correlated mortality in ciliates seems to be due to progressive deterioration of micronuclei. Several instances of chromosomal irregularities in old stocks have been reported for Paramecium (Dippell, 1955; Sonneborn and Schneller, 1960) and Tetrahymena (Nanney and Nagel, 1964; Wells, 1965).

A high mortality among exconjugants of Stentor has also been encountered by Burchill (1967) and by Harden and Holland (1968). However, both of these studies report the

survival of some exconjugant cells, as does the present study. These viable exconjugants appeared quite normal, and were able to proliferate, eventually producing clones. The fact that some exconjugant cells do survive is encouraging and should serve as a stimulus to seek the conditions necessary to increase viability.

In retrospect, the reported occurrence of mating types in Stentor should provide an experimental system with which to investigate inheritance in this genus. In addition, if conjugation can be brought under precise and stringent experimental control then biology would be provided with a most powerful tool toward analyses of very fundamental questions. Cross breeding could be combined with microsurgery so that one could ask, for example, whether the macronucleus from a mating type I cell, when transplanted to a mating type II cell, will cause the host cell now to become mating type I - again, will cytoplasm from a mating type I cell plus macronucleus from a mating type II cell give, in combination, a mating type II cell. The outcome of such experiments would provide valuable information on the relationships which exist between the nucleus and the cytoplasm.

SUMMARY

1. Two experimental methods which regularly yield large numbers of mating pairs were used to investigate conjugation in Stentor coeruleus. One method involves a particular culture technique, the other requires a mixture of different stocks.
2. Mating pairs appeared in the form of conjugation bursts, either induced by mixing certain stocks, or occurring spontaneously in some stock cultures and in clones produced by single cells isolated from them.
3. Spontaneous conjugation bursts, in the majority of cases occurred during a definite interval in the development of a culture, possibly due to the nutritional conditions within the culture.
4. Morphologically distinct pre-conjugator cells appear immediately before as well as during the initial stages of a conjugation burst. Mating pairs are formed by the union of pre-conjugators.
5. Mixing eight stocks in all possible combinations of twos and observing their subsequent response revealed they were separable into two complementary mating types.
6. The majority of mating pairs formed in mixtures were of the cross-mating type.
7. Contact between cells of differing mating type appears to be necessary for the initiation of a mating reaction.

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APPENDIX

Origin and History of Stocks

Stock C

This stock was obtained in August 1967 from Carolina Biological Supply Company (Burlington, North Carolina). It was collected in the vicinity of Burlington and had been maintained in culture for approximately 35 years.

Stock DF

This stock was isolated from a water sample taken from Ross Lake, British Columbia in March, 1968.

Stock S

This stock was obtained in June 1966 from Dr. Vance Tartar. It was collected from Stella Lake, Washington and had been maintained in culture since 1954.

Stock SC

This stock was obtained in February 1968 from Schettle Biologicals (P.O. Box 184, Stillwater, Minnesota). It was collected in the vicinity of Greensboro, North Carolina and had been maintained in culture for three years.

Stock ST

This stock was obtained in February 1968 from E.G. Steinhilber + Co. (P.O. Box 888, Oshkosh, Wisconsin). It was originally started from organisms belonging to Stock SC.

Stock T

This stock was obtained in August 1967 from the General Biological Supply House (8200 South Hoyne Ave., Chicago, Illinois). It was collected in the vicinity of Chicago and had been maintained in continuous culture for approximately 15 years.

Stock CH

This stock was obtained in October 1967 from Charles Harden (Science + Engineering Inc., 140 Fourth Ave., Waltham, Massachusetts). It was originally started from organisms belonging to Stock T.

Stock H

This stock was obtained in November 1966 from Noel De Terra (The Institute for Cancer Research, 7701 Burlholme Ave., Philadelphia, Pennsylvania). It was originally started from organisms belonging to Stock T.

Stock W

This stock was obtained from Wards Natural Science Establishment (P.O. Box 1712, Rochester, New York). It had been maintained in culture for three years, but its origin was not known.