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[ESSAY]

My Paramecium Study, Retrospect and Prospect

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I have often been asked why you studied *Paramecium*. I have never studied *Paramecium* itself but used it as the material for studying basic problems of life. In 1946, in the 2nd year of graduate study, I shifted my research material from multicellular organisms to the unicellular *Paramecium*. The reason was that if you study biology you should pursue basic principles to control every organism and for this purpose you should use a simple organism as the model system. I thought that *Paramecium* was a simple organism and did not know that much simpler organisms, *E. coli* and coli-phages, were already used to pursue the basic principles of life, since nearly all scientific informations overseas did not come in because of the war. In addition, I had a prejudice against bacteria and viruses that they were materials for medical science but not for basic biology.

Until the middle of 20th century, almost all fields of biology were of descriptive and comparative nature and only exception was genetics which established a basic law covering a wide range of organisms. To study genetics in these days, existence of sexuality was thought necessary and the sexuality in *Paramecium* was already reported by Sonneborn (1937). *Paramecium* was one of the major model systems for genetics in middle 1950th. This was the period of biochemical genetics when important materials for genetic analysis was moved from *Drosophila* and higher plants to eukaryotic microorganisms, though bacteria were out of focus to the geneticists because their sexuality was not discovered yet (Catcheside, 1951). In addition, genetics of *Paramecium* at that time was a character before the footlights, because of the kappa genetics developed by Sonneborn (1943). Kappa genetics was an essential topic in genetics chapter in many textbooks of introductory biology. Kappa particles were initially thought as a kind of genes contained in cytoplasm because they contained DNA. This was the time before the discovery of DNA in chloroplasts and mitochondria. Sonneborn (1950) proposed the plasmagene theory which was welcomed by those studying differentiation and development, who had a frustration by the fact that gene theory was unable to explain differentiation and development. The plasmagene theory, however, disappeared when the kappa particles were proved to be a kind of symbiotic bacteria (Preer, 1950). In 1946, as a graduate student majoring biology, I made up my mind to study

microbial genetics using *Paramecium*. To do this, it was necessary to have complementary mating types. I spent about 10 months for collecting *Paramecium* in different localities in Japan. Most abundant species of *Paramecium* collected in fields was *P. caudatum* at that time. I could find 4 pairs of complementary mating types. Two pairs were later identified the same as those Gilman discovered already (Gilman, 1941) and the other two were new. When cells of complementary mating types were mixed, large clumps of mating agglutination occurred. I was so startling at this observation that I decided to study this spectacular event. The first problem I wanted to attack was to see whether the agglutination was induced by some soluble substance excreted from the cells, as in the case of sperm agglutination by egg water I studied before. I tried to put cells of one mating type into cell-free fluid of the complementary mating type. Nothing happened. Soluble mating inducing substances were later found not in *Paramecium* but in hypotrichous and heterotrichous ciliates (see Miyake, 1996).

Since no agglutination-inducing factor was found in culture fluid, I tried to find the factor on the cell surfaces. At this time, I found a brief report of inducing conjugation by formalin-killed cells of the complementary mating type in "Biological Abstracts" which was an only available window to see the progress of biological researches in foreign countries. This was a report with *Paramecium aurelia* (Metz, 1947) and I repeated this experiment with *P. caudatum*. Not only this repetition was successful but I found an interesting difference between complementary mating types. When one mating type was formalin-killed and mixed with living cells of the complementary type, mating agglutination occurred but the opposite combination of killed and living mating types gave no agglutination. Later, this difference of mating types was found to be due to the difference of inactivation concentrations of formalin between complementary mating types. This was the first chemical difference of sex (mating type) found in *Paramecium* (Hiwatashi, 1950).

Then, I tried to identify chemical properties of the mating agglutination molecules by enzymatic inactivation of the killed cells. While I was wondering how to get crystalline preparations of proteases and other enzymes which were then difficult to obtain in Japan, I found that the same kind of experiments was already reported with *P. aurelia* (Metz & Butterfield, 1951). Since then, many laboratories tried to iso-

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late the mating agglutination molecules (called the mating substances) for more than decades. However, the simplest preparation to induce mating agglutination thus far obtained was not the mating substances in molecular form but a kind of membrane vesicles (Kitamura & Hiwatashi, 1976). Recently, however, monoclonal antibodies which inhibit mating reactivity of only one type of complementary mating types were isolated (Azuma *et al.*, 1996). This may predict successful isolation of mating substances before long.

Studying mating agglutination and conjugating pair formation, I discovered two important facts which contradict to those already reported in other species of *Paramecium*. One is that only the cilia covering the ventral surface of *Paramecium* have the mating reactivity. This was proved by the experiment that when formalin-killed mating reactive cells were cut into ventral and dorsal sides, only the ventral side reacted with living cells of the complementary mating type (Hiwatashi, 1961). This was later confirmed to be also true in *P. bursaria* by the experiment that when detached cilia from mating reactive cells were mixed with living cells of the complementary mating type, the former adhered only to the ventral surface of the latter. The other one is that the ciliary mating agglutination occurs strictly between complementary mating types but subsequent pair formation occurs not only between complementary mating types but also between the same mating types. This was proved by marking different mating types by vital staining dyes, neutral red and Nile blue sulfate (Hiwatashi, 1951). When studying mating agglutination and conjugating pair formation, I was often disturbed by occurrence of selfing conjugation in one mating type, because in studying mating agglutination and conjugating pair formation, spontaneous occurrence of selfing conjugation destroyed the schedule of experiment. I wanted to know how mating types are controlled genetically because I thought that if I know genetics of mating type, I might exclude the spontaneous occurrence of selfing conjugation, though occurrence of the selfing conjugation was later proved to be due to spontaneous occurrence of mating type change (Hiwatashi & Myohara, 1976).

In *P. aurelia* species complex, Sonneborn already reported that mating types are controlled cytoplasmically or caryonidally (see Sonneborn, 1947). My question was whether mating type of *P. caudatum* is also controlled in the same way. When cross breeding analyses were made with complementary mating types of *P. caudatum* the result was neither cytoplasmic nor caryonidal inheritance but synclonal inheritance (two exconjugant clones from a pair show the identical phenotype) which suggested a direct gene control. When I was at this stage of my research, I had an opportunity to go to Sonneborn laboratory in the Indiana University supported by the Rockefeller Foundation. I continued to study mating type genetics of *P. caudatum* in Sonneborn Lab because he had a strong interest in my results suggesting direct gene control of mating type and encouraged me to extend it. When I tried to get segregation data in F2 and backcross, I confronted with difficulty of poor survival after conjugation. At first, I thought that some environmental conditions were guilty. My every

effort for nearly 10 months of changing environmental conditions, however, failed. Eventually, main cause of the poor survival after conjugation was found to be due to aging of the stocks used. It would be interesting to mention that none in the Sonneborn Lab suggested me the possible cause of poor survival to be the clonal aging. The person who suggested me clonal aging to be the cause of poor survival after conjugation was a *Tetrahymena* worker. Everyone in the Sonneborn Lab was working with *P. tetraurelia* in which frequent occurrence of autogamy prevented the cells from advancing aging and thus experienced no poor survival after conjugation.

In 1962, I was asked to return to my university in Japan, though I wanted to stay in the U.S. much longer. One merit of returning to Japan for me was that I would have young students in my laboratory every year. I encouraged those students in doing free discussion. This was the best thing I learned in Sonneborn Lab.

As compared with *P. aurelia* complex, *P. caudatum* has two inferior points as the genetic material, lack of autogamy and existence of long immature period (about 50 fissions) after conjugation. The lack of autogamy was overcome by artificial induction of autogamy by interrupting conjugation by proteases (Tsukii & Hiwatashi, 1979). To make the long immature period shorter or nothing, I first tried to culture immature cells in the medium containing brei of mature cells. This was failed. Then, one of my graduate students tried to inject cytoplasm of mature cells into immature cells. This was also failed. I happened to suggest him to see what would occur if immature cytoplasm was injected into mature cells. Unexpected result was obtained. The injected cells changed to immaturity (Miwa *et al.*, 1975). The effect was the same when soluble fraction of the immature cells was injected. This fraction was purified and found to be a heat-labile protein of about 10,000 daltons (Haga & Hiwatashi, 1981). This protein was named as immaturin and cited even in papers outside ciliate genetics. The orthodoxical way to avoid long immaturity after conjugation must be isolation of mutants with shorter or no immaturity period. In mutant isolation, success or failure often depends on how to screen the mutant we want. By using erythromycin resistant cytoplasmic marker, one of my graduate students succeeded in isolation of mutants having shorter period of immaturity (called early mature mutants) in two different loci. These two mutants were both dominant and had additive effect in shortening the immature period (Myohara & Hiwatashi, 1978).

After the success of the immaturin study using microinjection technique, this technique came into fashion in my laboratory. Because of the large size of *P. caudatum*, it is much easier to use microinjection in this species than in *P. aurelia* complex and *Tetrahymena*. Sometimes, a glass needle of as large as 10 micrometer tip diameter is able to insert into living cells without injury (Harumoto & Hiwatashi, 1982). In addition to the large size of the cell, the fact that the micronucleus of *P. caudatum* is compact type and easy to be observed in living cells makes it easier to transplant from cell to cell. Several important studies were made by this technique. One of them

was to answer the question whether the synkaryon in *Paramecium* conjugation is undetermined neutral nucleus or determined germinal nucleus (micronucleus). When the synkaryon was transplanted into an amiconucleated vegetative cell, it had an ability to undergo meiosis, although it never passed through the special germ-nuclear determining process after conjugation (Harumoto & Hiwatashi, 1982). Another experiment was to answer the question whether germinal micronucleus ages with clonal aging. When micronucleus in cells of very aged clone with very low fission rate and no survival after conjugation was transplanted into amiconucleate cells of very young clone, some of the young cells with old micronucleus not only divided normally but also showed a high survival after conjugation (Karino & Hiwatashi, 1984). In addition to the nuclear transplantation, removal of nuclei by sucking with microneedle was also useful technique to obtain important results. Two nuclear behaviors were proved to depend on so-called positional information. One is the survival or degeneration of meiotic products. When a meiotic product located at the paroral region was removed, one of the remaining degenerating three nuclei moved to the paroral region and survived (Yanagi, 1987). This proved that the paroral region has the rejuvenating effect on the meiotic products once determined to degeneration. Another example of the positional information revealed by this technique was the determination of micro- and macronuclei at the 3rd postzygotic division. Various removal and transplantation experiments of postzygotic division products show that anterior and posterior localizations of the 3rd postzygotic division products determine the differentiation of germinal and somatic nuclei (see Hiwatashi & Mikami, 1989).

When microinjectionists increased in my laboratory, they were often invited to ciliate laboratories abroad to cooperate. Once when I gave a lecture in some university in China, one student in the audience asked me the question, "is microinjection difficult?" I answered this student, "it may not be difficult for you because you have grown up using chopsticks in every meal".

There are many interbreeding groups in *Paramecium*. These groups were first called mating groups, then varieties and later syngens. Syngen means "generating together". This term was coined by Sonneborn (1957) for the following reason. The mating groups should be species but taxonomical species must be identified only by description. Identification of *Paramecium* mating groups at that time, however, needed living stocks of standard mating types and was unable to do only by description. Later, mating groups (syngens) of *P. aurelia* complex became identifiable by marker isozymes without standard living stocks (Sonneborn, 1975). Thus, *P. aurelia* syngens are now taxonomical species with binominal Latin names. In the beginning, I thought that *P. caudatum* syngens might be like *P. aurelia*, where intersyngenic crosses, if possible, are lethal, or sterile even if F1 could survive. To try intersyngenic crosses in *P. caudatum*, convenient genetic markers should be necessary. Fortunately, in 1978, very convenient genetic markers became available. These are behav-

ioral mutants isolated by one of my research associates. She was successful in isolating more than a dozen behavioral mutants (Takahashi, 1979). Among them, the behavioral mutants named CNR (caudatum non-reversal) was the convenient marker to distinguish homotypic and heterotypic conjugating pairs. By using this genetic marker and mixing two pairs of complementary mating types belonging to different syngens, one of my graduate students succeeded in isolation of intersyngenic conjugating pairs. To my great surprise, the intersyngenic hybrids of *P. caudatum* were neither lethal nor sterile. By extensive analyses of intersyngenic cross breedings, we established the three gene hypothesis of mating type and syngen specificities (Tsukii & Hiwatashi, 1983). Thus, in *P. caudatum*, mating groups were found not to be 100% sexually isolated and should not be called syngens in its original meaning. When I sent the first draft of our results to Sonneborn, he had a special interest in it and wrote us that he introduced our results in his laboratory seminar spending one and half hours. To my great regret, this paper was the last one for which we received his very kind and helpful comments. Now, there are the words, *Paramecium* has become an "endangered genetic species". This is the words cited by John Preer, the successor of Sonneborn in Indiana University, in his essay published in *Genetics* (Preer, 1997). Comparing with the big five (*E. coli*, yeast, *Drosophila*, *C. elegans* and mouse), protozoan ciliates including *Paramecium* have now very few number of investigators. Among ciliate workers, majority at present seems to be those using *Tetrahymena*. *Tetrahymena* seems more convenient material than *Paramecium*, especially for those studying biochemical and molecular biological works. Probably, *Paramecium* is the material for those who like to observe swimming living cells under the binocular microscope rather than for those who like to see DNA bands on the gel. Now it is the age of molecular biology, and so far *Paramecium* is not among the main characters advancing molecular biology. However, does this predict extinction of *Paramecium* as genetic species? No, I don't think so, because I can see many attractive and important subjects in *Paramecium* genetics.

In *Paramecium* clones, the life span is counted not by physical time but by a biological time, the number of fissions. The length of sexual immaturity (including interautogamous intervals called autogamy immaturity) is also determined by the number of fissions (see Takagi, 1988). There is a possibility that the biological time by the number of fissions is actually the number of DNA replications in macronucleus (Mikami & Koizumi, 1983). If this were true for length of immaturity and life span, it would be very interesting to study molecular mechanisms for counting DNA replications.

Binuclearity of germ and somatic nuclei in the cells of ciliates was once taken that ciliates are exceptional organisms. There is, however, an old saying, "treasure your exceptions". The most valuable "exception" seen in *Paramecium* as well as in other ciliates seems to be the exconjugant cell. In exconjugant cells (also in exautogamous cells), three different nuclear processes occur within a single cell. Those are

2C and 4C cycles of DNA synthesis in micronucleus, continuous synthesis of DNA up to more than 100C in macronuclear anlagen and degradation of DNA in old macronuclear fragments. How these three different processes are controlled within a single cell must be an extremely interesting problem. What I can hardly omit to mention here is the so-called "macronuclear inheritance". This was first discovered in a mutant of serotype in *P. tetraurelia* which showed a cytoplasmic pattern of inheritance. The mutant was found to be due to a defect in DNA processing and this defect to be controlled by DNA in the old macronucleus (Mayer & Forney, 1996; Mayer *et al.*, 1998). This suggests that the three different nuclei in the exconjugant cell, new micro- and macronuclei and old macronuclear fragments, are not independent but are interacting each other. Probably, many other genic interactions are waiting for analyses by younger *Paramecium* workers.

In conclusion, I would like to say that *Paramecium* is a rich mine of scientific investigations and so, we should not let it extinct as genetic species.

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