Induction of Conjugation by Cell-Free Preparations in Paramecium multimicronucleatum

Abstract. *A cell-free preparation composed mainly of cilia, obtained by treatment with $K_2Cr_2O_7$ from stocks of* Paramecium multimicronucleatum *(syngen 2) pure for mating type, can regularly induce conjugation in intact animals of the opposite mating type.*

In *Paramecium*, conjugation is normally initiated by the mating reaction (*1, 2*). The latter is a loose but conspicuous mass agglutination that occurs between animals of complementary mating type under appropriate conditions. The agglutination continues for some time, usually about an hour, until pairs unite more intimately at their anterior ends (holdfast union) and then in the paroral region, yielding typical conjugant pairs.

Earlier works showed that the agglutination is not mediated by substances dissolved in the medium but is caused by direct interaction of paramecia surfaces, probably the surfaces of cilia (*1, 3, 4*). Detached cilia of mating-reactive animals specifically adhere to intact animals of the opposite mating type (*5, 6*). Therefore, the substances participating in agglutination are considered to be on the cilia.

Dead paramecia can activate and induce conjugation in living paramecia of the opposite mating type after agglutination with the living animals (*4, 7*). These results suggest that the substances responsible for the trigger reaction which activates paramecia to conjugate are also on the surfaces of paramecia, probably on the cilia.

This view is supported by the results presented here which show that cell-free preparations composed mainly of cilia can regularly induce conjugation in intact animals of the opposite mating type.

For the present work it was necessary to use stocks which remain constant for mating type. Most stocks of *Paramecium multimicronucleatum*, syngen 2, have a circadian cycle of mating-type change, but some have no such cycles—that is, they do not change mating type while they reproduce by binary fission except on very rare occasions (*8*). From Barnett's (*8*) stocks, cycleless stocks of each mating type were derived by experimental breeding (*9*). Among them, the following were used in this study: CH204, CH210-1, and CH551, all of mating type III; and CH202, CH203, CH210-2, and CH325, all of mating type IV.

The cell-free preparation composed predominantly of cilia was obtained as follows. Paramecia were cultured in 3000-ml erlenmeyer flasks at 25°C, baked lettuce infusion inoculated with *Aerobacter aerogenes* being used as the nutritive medium. This medium was prepared according to Sonneborn's method (*10*) with two modifications. First, sodium phosphate buffer solution was used, instead of $Ca(OH)_2$, to adjust the *p*H. Ten milliliters of sterilized sodium phosphate buffer of *p*H 6.8 (200 m*M*) was added to each liter of lettuce medium before use. Second, 1.3 ml of canned vegetable juice, Meiji V7, was added to each liter of the lettuce infusion before autoclaving. Fission rate during culture was adjusted to about one fission per day by controlling the amount of culture medium added daily. The paramecia were concentrated by mild centrifugation to approximately 15,000 animals per milliliter and were kept at 25°C. After 12 to 16 hours, their mating reactivity was examined, and, if they were strongly reactive, the paramecia suspension was mixed with 200 m*M* of $K_2Cr_2O_7$ solution so that the paramecia would be suspended in 20 to 30 m*M* of $K_2Cr_2O_7$ solution. Only a relatively narrow range of $K_2Cr_2O_7$ concentrations, which depended on the density of the paramecia, culture method, and stock gave satisfactory results. In these solutions, paramecia died within a few hours at 25°C. Just before the paramecia died, they discharged many trichocysts, and, at this moment or immediately after it, most of the cilia dropped off, but the animal bodies did not disintegrate. After all the paramecia had died, the suspension was stirred gently and most of the dead bodies were precipitated by mild centrifugation. The supernatant was then filtered through a sheet of filter paper (Toyo filter paper No. 2). By a single filtration all the remaining dead bodies and virtually all trichocysts were removed, the filtrate being a reasonably pure suspension of cilia. Cilia were precipitated by centrifugation (6500*g*, 30 minutes). Precipitated cilia were washed by repeated centrifugation in 40 m*M* KCl buffered by sodium phosphate (*p*H 6.8, 10 m*M*). In this way the cilia obtained from approximately 2,500,000 paramecia were collected in 10 ml. There was, however, a considerable loss in the procedures, especially during filtration. The suspension of cilia is called "the cilia fraction."

Instead of washing, the $K_2Cr_2O_7$ could be removed by dialysis, after collecting the first precipitate of cilia with a small amount of fluid. The suspension was dialyzed against 40 m*M* KCl buffered by sodium phosphate (*p*H 6.8, 10 m*M*) in a refrigerator, until the cilia suspension became colorless.

The cilia fraction thus obtained was composed predominantly of cilia. A vesicle was attached at one terminus of most cilia. Other microscopically visible constituents were trichocysts, bacteria, and small granules. Trichocysts and bacteria were relatively few in number. The small granules, some of them probably derived from the culture medium, were more numerous, and there appeared to be several different kinds.

The cilia fractions obtained from stocks CH210-1 and CH551 were strongly effective in inducing conjugation in living animals of stocks of the complementary mating type (CH202, CH203, CH210-2, and CH325), but completely ineffective on stocks of the same mating type (CH204, CH210-1, and CH551). In agreement, the cilia fraction obtained from stock CH210-2 was effective in inducing conjugation in stocks CH204 and CH210-1, but completely ineffective on stocks CH202, CH203, and CH210-2. The conjugation-inducing activity of the cilia fraction is, therefore, specific for the mating type—that is, it induces conjugation only in living animals of the opposite mating type.

One-tenth of a milliliter of the cilia fraction of stock CH210-1 or CH551 regularly induced 10 to 80 percent conjugation in approximately 3000 animals of mating type IV suspended in 1 ml at 25°C. Control animals to which no cilia fraction was added did not conjugate at all, nor did they when suspended in the supernatant obtained by centrifuging the cilia fraction.

When the cilia fraction of mating type III was added to living animals of mating type IV, many of the latter stuck to each other. If enough of the cilia fraction was added, the sticking began in a few seconds like a mating reaction. This sticking was probably caused by the adherence of cilia of

mating type III to living animals of mating type IV, thus making these animals capable of sticking to others. With a phase contrast microscope, it was observed that cilia in the cilia fraction of mating type III adhered to animals of mating type IV. Cilia did not adhere evenly to the whole area of the paramecia, but adhered mainly to the oral side. This result agrees with the observations that in *Paramecium caudatum* (*11*) and in *Paramecium bursaria* (*6*) only the cilia on the oral side of the paramecia have mating reactivity.

After 40 to 60 minutes of sticking together, holdfast pairs appeared. They increased in number, and a short time later typical conjugant pairs appeared.

Conjugation induced by the cilia fraction appeared to be normal. Each pair was tightly united at the oral region. In each conjugant, micronuclear divisions and a typical skein formation of the macronucleus occurred. In exconjugants, the old macronuclei broke down into small fragments, and new macronuclear anlagen were formed. After reorganization, exconjugants could establish viable clones. Because these phenomena are characteristic of conjugation, the induced unions will be referred to as conjugation although the occurrence of reciprocal fertilization was not ascertained.

In the reciprocal combination, that is, the cilia fraction of mating type IV and living animals of mating type III, the induction of conjugation was less successful. The highest percentage of conjugation ever obtained in this combination was only 2 percent, suggesting that the substance involved in mating type IV is less stable under these conditions.

The cilia fraction gradually lost its conjugation-inducing activity. It became completely ineffective in 2 or 3 days at 25°C. In a refrigerator the decline occurred more slowly. At 2° to 3°C, the cilia fraction retained strong activity for up to 10 days or more. To preserve activity for a longer time, the best method known at present is to freeze the cilia fraction with glycerol. According to this method, 1 to 2 ml of glycerol is mixed with 8 to 9 ml of the cilia fraction and the mixture is kept in a deep freeze (about −20°C). Before use, glycerol is removed by dialysis against 40 m*M* KCl solution buffered by sodium phosphate (*p*H 6.8, 10 m*M*), after thawing at room temperature. Freezing and thawing without glycerol greatly decrease the activity, unless the cilia fraction is frozen very quickly.

If paramecia are killed in $K_2Cr_2O_7$ solutions of higher concentrations (up to 80 m*M*) than those used in the method described above, the cell-free preparation obtained does not have a strong conjugation-inducing activity. However, paramecia killed by such strong solutions and washed free of $K_2Cr_2O_7$ can strongly adhere to living animals of the opposite mating type and can induce conjugation in them. It was observed that most of the cilia are not detached from these dead paramecia, in agreement with the suggestion that the conjugation-inducing activity is associated with cilia.

The possibility that any dissolved substance derived from paramecia is responsible for the conjugation-inducing activity of the cilia fraction is excluded by two observations: (i) the cilia-free supernatant obtained by centrifuging the cilia fraction has no activity, and (ii) the precipitate retains strong activity after repeated washing. Therefore, the activity must be associated with particulate materials in the cilia fraction.

Among the microscopically visible constituents of the cilia fraction, trichocysts and bacteria can hardly be responsible for the activity. They are both few in number. In the $K_2Cr_2O_7$ solution, paramecia discharge many trichocysts. After the removal of dead bodies, a supernatant containing the cilia but relatively much richer in trichocysts can be obtained by mild centrifugation. After dialysis, this supernatant can induce conjugation in living animals of the opposite mating type, but its conjugation-inducing activity is not higher than that of the cilia fraction which is much poorer in trichocysts. If trichocysts alone were the active particulate materials, the supernatant should have been many times more effective than the cilia fraction. Since it was not, the trichocysts can be excluded. The bacteria-rich culture medium in which paramecia of one mating type have lived has no conjugation-inducing activity on the opposite mating type, indicating that bacteria alone can not be responsible for the activity.

Most of the small granules found in the cilia fraction are probably derived from the culture medium. Since the culture medium in which paramecia of one mating type have lived is not effective in inducing conjugation, such granules derived from the culture medium alone can not be responsible for the activity. However, some of the small granules are probably derived from paramecia during the treatment with $K_2Cr_2O_7$. Although paramecia killed by $K_2Cr_2O_7$ do not disintegrate, some of them are partly broken. The possibility that such granules derived from paramecia might be responsible for the activity of the cilia fraction cannot be excluded at present and should be examined further. However, considering the great number of cilia in the cilia fraction, the relatively small number of granules, and the strong conjugation-inducing activity of the cilia fraction, it may be tentatively concluded that cilia are essential for the conjugation-inducing activity of the cilia fraction.

This conclusion is not an unexpected one. The importance of cilia in the conjugation of paramecia has long been recognized. Sonneborn (*1*), as soon as he discovered the mating reaction as a prerequisite to conjugation, realized that the reaction takes place between paramecia surfaces, probably between cilia. Metz (*5*) obtained detached cilia of mating-reactive *Paramecium aurelia* killed in formalin and showed that the cilia adhered to intact animals of the opposite mating type. Cohen and Siegel (*6*) demonstrated that detached cilia of mating-reactive *Paramecium bursaria* (prepared by mechanical disruption of paramecia by repeated ejection through a medical syringe) specifically adhere to mating-reactive animals of the opposite mating type. These may constitute sufficient evidence to conclude that cilia participate in the mating reaction—that is, the initial agglutination.

Metz (*4*) found that *Paramecium aurelia* killed by certain methods not only adhere to living animals of the opposite mating type but also can activate them to form pairs (pseudoselfing). Comparable results were obtained by Hiwatashi (*7*) in *Paramecium caudatum*. These results, together with the widely accepted view that the mating reaction is a necessary prerequisite to normal conjugation, suggest that cilia are also involved in the activation. However, using whole dead animals, these investigators could not exclude the possibility that components of paramecia other than cilia participate in the activation. It is possible to postulate that the role of the mating reaction is merely to hold potential conjugants close together and that the trig-

ger reaction for activation is not an interaction between the cilia taking place while animals of complementary mating type are in loose contact. In the chemical induction of conjugation, paramecia of the same mating type conjugate with each other without showing a prior mating reaction, indicating that the agglutinative reaction is not an absolute prerequisite for the occurrence of conjugation (*12*). Therefore, the conclusion that cilia participate in the mating reaction and the demonstration that dead *P. caudatum* can induce living animals of the opposite mating type to conjugate do not necessarily mean that cilia can activate paramecia to conjugate.

The experiments reported here make this point clearer, for they demonstrate that the cell-free preparation predominantly composed of cilia can induce conjugation in paramecia of the opposite mating type; they thus indicate that isolated cilia not only adhere to paramecia of the opposite mating type but can also activate them to conjugate.

Since both agglutination and activation should have a material basis, substances participating in agglutination, and others participating in activation, may be postulated. The former have been called mating type substances and are believed to be proteins (*5*, *6*). Neither they nor activation substances have yet been extracted in soluble form.

These two kinds of substances may be identical as Metz (*5*) postulated. According to his postulation, the interaction between mating type substances themselves is the trigger reaction which activates paramecia to conjugate. The present experiments do not prove this but they do at least indicate that both phenomena are due to substances on the same organelles, the cilia.

The problem would be solved if these substances could be extracted and compared. Such studies may be facilitated by using the method reported here, since it yields cell-free preparations which possess strong activities of both kinds, specific adhesion to cells of complementary type and activation for conjugation.

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Mitomycin C: Effect on Ribosomes of Escherichia coli

Abstract. *Sucrose-gradient analyses of cell-free extracts of* Escherichia coli *B show that the sedimentation patterns are markedly affected by exposure of the cells to mitomycin C; 50S subunits are progressively degraded in the cells after 30 minutes exposure. The 30S subunits, although affected, are less sensitive to the antibiotic than the 50S subunits. Incorporation of uracil-2-C^{14} into the ribosomal particles by the treated cells is markedly reduced by exposure.*

Several reports (*1*, *2*) suggest that the antibiotic mitomycin C (MC) has a specific effect on cellular DNA. Other studies indicate that its effect is not entirely limited to DNA (*3*, *4*). Kersten and Kersten (*5*) have found that MC causes primarily the breakdown of RNA and suggest that breakdown of DNA may be a secondary reaction. Our studies on the mode of action of MC in *Escherichia coli* also show that the inhibitory action is more complex than first believed. We now report that ribosomes in cells of *E. coli* B are degraded during exposure to MC. Evidence is also presented indicating that the ability of ribosomes to incorporate uracil-2-C^{14} is impaired in exposed cells.

Exponentially growing [in a glucose-synthetic medium (*6*)], cells of *E. coli* B were washed with 0.05*M* phosphate buffer, *p*H 7.0, suspended in 100 ml of the same culture medium at a concentration of 5×10^8 cells per milliliter, and incubated at 37°C in the presence of MC, with aeration. After treatment the cells were harvested, washed twice with 0.01*M* tris-HCl buffer (*p*H 7.3), and suspended in 5 ml of the same

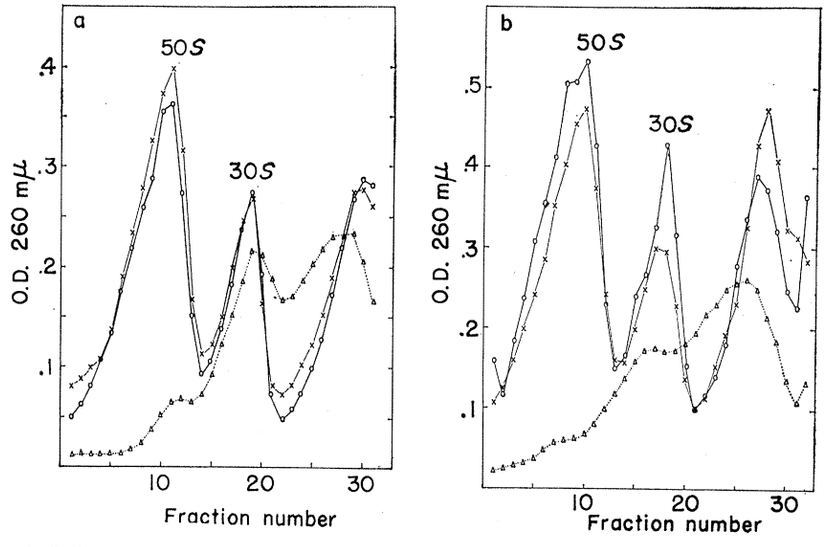

Fig. 1. Sedimentation profiles of extracts of *Escherichia coli* B obtained after 60 minutes (*a*) and 90 minutes (*b*) treatment with MC. Cells were incubated in the absence of MC as controls (o——o), in the presence of MC at 0.1 μg/ml (×——×), and in the presence of MC at 5.0 μg/ml (Δ . . . Δ). Crude extract (0.5 ml) in 0.01*M* tris-HCl buffer containing $10^{-4}M$ magnesium acetate, *p*H 7.3, was layered on the top of 4.5 ml of sucrose in a linear density gradient (5 to 20 percent sucrose in 0.01*M* tris-HCl buffer and $10^{-4}M$ magnesium acetate, *p*H 7.3) in cellulose centrifuge tubes. Each preparation was centrifuged at 100,000*g* for 120 minutes at 4°C in a swinging-bucket rotor (SW 39) in a Spinco model L ultracentrifuge. Each tube was then punctured at the bottom, and 10-drop fractions were collected and diluted to 3.0 ml for optical density measurements at 260 mμ.