Sexual Reproduction of *Chlamydomonas* as Affected by Ionic Balance in the Medium*

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Among environmental factors inducing sexuality in a green alga, *Chlamydomonas moewusii* var. *rotunda*¹⁾, depletion of assimilable N in the medium was found to be one of the important²⁾, and furthermore, light appeared to promote the mating activity³⁾. Although the mechanism of inducing sexuality is yet unclear, the observations agree with the general conclusion of many reports in the literature, beginning with Klebs⁴⁾, that the presence of light and the depletion of nutrient induce sexuality in a number of diverse algae⁵⁻⁸⁾. On the other hand, however, distilled water, in spite of its lack in N, does not make a medium more suitable for yielding abundant sexually functional cells than N-free synthetic medium⁹⁾. Other factors besides N in medium must therefore be involved in the induction of sexual activity. This paper concerns experiments performed to reveal the effect of some other factors, especially the ionic balance in the medium, on the sexual reproduction of this alga. An observation on the dedifferentiation of sexuality in a medium with assimilable N is also described.

Material and Methods

Organism. *Chlamydomonas moewusii* Gerloff var. *rotunda* Tsubo¹⁾ (used as *C.* sp. 24 in previous experiments^{2,9)}) was originally isolated by the present author from Japanese soil. The organism is heterothallic and isogamous; two mating types are arbitrarily designated as + and -. This alga grows on an inorganic medium M*** under continuous illumination (ca. 2,000 lux) with fluorescent tubes (warm white) at 23°.

Mating of cells and estimation of sexual activity. When cells mate, they make *vis-à-vis* pairs; two cells are connected with each other by a protoplasmic bridge at their apical ends²⁾. The mating pairs swim for ca. 6 hrs., withdraw their flagella and fuse to form zygotes. Since sexually functional cells are morphologically analogous to non-sexual cells, the appearance of sexuality can only be recognized by the ability of producing mating pairs in the mixture of the two mating types. Therefore, for estimating the sexual activity, a drop of the cell-mixture was transferred onto a slide glass and fixed by a drop of acetocarmine, and then the frequencies of mating pairs and zygotes were counted under the microscope; a zygote was counted as equivalent to two gamete-cells.

In the present experiment for inducing sexuality, the following method²⁾ was ap-

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*** Composition of medium M: NH_4NO_3 , 400 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg.; KH_2PO_4 , 100 mg.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg.; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.39 mg.; H_3BO_3 , 0.6 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 mg.; $\text{H}_3\text{PO}_4 \cdot 12\text{MoO}_3 \cdot 6\text{H}_2\text{O}$, 0.003 mg.; $\text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O}$, 200 mg.; distilled water, 1,000 ml.

plied; vegetative cells of each mating type growing in medium M in the light were separately washed with distilled water, and equal amounts of each kind of cells were mixed together in a test solution of given salt concentration. The mixture was illuminated for 10~15 hrs., so that all the sexually functional cells produced in the mixture could accomplish mating. The mating frequencies shown in the following experiments represent the mean value of 2 to 4 parallel runs. All the cultures examined in this study were performed with a cell density of $10^5 \sim 10^6$ cells/ml.

Results

Dedifferentiation of sexuality in the presence of assimilable N: As reported in a previous experiment³), when growing cells in a liquid medium were transferred to a N-free medium (M-N) and illuminated, sexual reproduction was induced, i.e. if the starved cells of each mating type were mixed together, cellular agglutination occurred, and a lot of mating pairs were eventually released; the growing number of mated gametes reached a maximum in about 2.5 hrs. after mixing (Fig. 1); the mating *per se* did not seem to require light.

In an experiment to demonstrate dedifferentiation of sexuality in the presence of assimilable N, illuminated gamete-cells of each mating type, obtained in M-N medium were separately inoculated into the following 3 kinds of media, i.e. M-N supplemented with NaCl, NH_4Cl , and NaNO_3 to a final concentration of 5 mM. each. All the cultures were placed in the light. At time intervals, equal amounts of the complementary mating types in each medium were mixed together and kept in the dark in order to avoid further development of mating activity by the light³). After 2.5 hrs., mated pairs were counted under the microscope. As shown in Fig. 2, sexual activity of the cells incubated in the presence of assimilable N was evidently decreasing. Neither cell division nor appreciable enlargement of the cells was noticed during the 5 hrs. Nevertheless, when sexually functional cells of each mating type were plated onto M-agar, corresponding to cell counts in the original suspension, colonies were produced. This finding suggests that, if the gamete-cells were not allowed to mate

Fig. 1. Number of mated gametes formed on mixing two mating types of *C. moewusii* var. *rotunda* in the light or in the dark (conditions of experiment, see text).

Fig. 2. Decrease in sexual activity in nitrogenous medium (salt concentration, 5 mM. each in M-N medium; conditions of experiment, see text).

and transferred to growing conditions, they could multiply as vegetative cells.

Thus, it is obvious that the assimilable N inhibits both gamete-formation²⁾ and/or mating of gametes. If the agglutination of gametes is supposed to be caused by the surface reaction of flagella^{9,10)}, the ionic constitution of medium might specifically influence this reaction, for instance, by interfering with the surface phenomenon in the presence of NH_4^+ or NO_3^- . On the other hand, the presence of N in the medium might have influenced the development of potential sexual activity in the cell on same nutritional ground. To elucidate the problem, the following experiments were planned to compare the effect of various ions on the induction of sexuality with that on the mating of gametes.

Induction of sexuality in single salt solution: Growing cells of both mating types were washed, mixed together and inoculated so as to make a population of 10^6 cells/ml. in each salt solution given in Table 1. Estimation of sexuality appeared in each solution was carried out after 15 hr.-incubation in the light. As shown in Table 1, sexuality in distilled water was less. Such cations as K^+ , Na^+ , and Mg^{++} promoted induction of sexuality. The depressing effect of NO_3^- on the activity of Na^+ and Ca^{++} is also clearly shown. Cells cultured in solutions of a higher concentration (50 mM.) of each salt were also examined, but all of them were more or less injured.

Saito¹¹⁾ described that ascus- or ascospore-formation of *Zygosaccharomyces major* was induced by the presence of such cations as Na^+ , K^+ , Mg^{++} , and Ca^{++} , added separately, or simultaneously as van't Hoff solution*. Therefore, the induction of sexuality in *Chlamydomonas* was examined with various concentrations of van't Hoff solution. The same procedures of incubation and testing were adopted for the use of van't Hoff solution. The increasing activity of sexual reproduction induced in van't Hoff solution up to 5 mM. might have been caused by the osmotic balance of the individual component ions. Therefore, the inductions of sexuality in various media lacking Na, K, Ca, Mg, S, or P were compared. For this experiment, 6 kinds of incubation media were prepared by mixing 2 ml. each of 4 stock salt solutions selected out of the following 7; MgSO_4 , MgCl_2 , KCl , CaCl_2 , K_2HPO_4 , Na_2HPO_4 and NaCl (5 mM. each). Fig. 4 shows that none of these 6 ions was indispensable for the induction of sexuality. The slightly less appearance of sexuality in the lack of K^+ or Ca^{++} might presumably have resulted from the ionic unbalance in the medium, especially since these two cations have strong antagonistic activities for controlling

Table 1. Frequencies (%) of mated gametes produced in inorganic salt solutions after 15 hr.-incubation in the light.

	0.5 mM.	5 mM.		0.5 mM.	5 mM.
LiCl	0	0	Na_2SO_4	12.6	45.5
KCl	19.8	42.1	MgSO_4	39.9	47.7
NaCl	15.8	27.8	$(\text{NH}_4)_2\text{SO}_4$	1.4	1.2
MgCl_2	23.3	39.7	NaNO_3	12.4	2.5
CaCl_2	6.7	8.5	$\text{Ca}(\text{NO}_3)_2$	3.7	3.4
BaCl_2	0	0	KH_2PO_4	61.6	64.8
NH_4Cl	0.6	1.4	$(\text{NH}_4)_2\text{HPO}_4$	0	0
Distilled water		6.8			

* One molar van't Hoff solution contains the following salts in 115.8 liters of water: 100 M. NaCl, 2.2 M. KCl, 2.0 M. CaCl_2 , 3.8 M. MgSO_4 , 7.8 M. MgCl_2 .

Fig. 3. Effect of concentrations of medium on induction of sexual activity (van't Hoff solution).

Fig. 4. Test of nutritional requirements for induction of sexuality (conditions of experiment, see text).

cell permeability, or for changing the physical state of protoplasm^{12,13}). The following experiment also supports this view.

Ionic interaction on induction of sexuality: The induction of sexuality was examined with incubation media of various ratios of KCl/CaCl₂, KCl/MgCl₂, or MgCl₂/CaCl₂. As shown in Fig. 5, the degree of induction of sexuality, as measured by the frequency of mated gametes, was always higher when two of the tested ions had been simultaneously present in the incubation medium, the best result being obtained in a mixture of KCl and CaCl₂ (5 mM.: 5/8 mM.), and also in the mixtures containing various ratios of KCl and MgCl₂. Although a similar increase in sexual induction was obtained in the mixtures of MgCl₂ and CaCl₂, the mixture was less effective in this respect than the other two combinations mentioned above.

Fig. 5. Induction of sexuality as affected by the ionic interaction (conditions of experiment, see text).

Mating activity of sexually induced cells as affected by various salts: In the experiment described above, it was clearly shown that a high sexual activity was induced in cells incubated in the light in a solution containing ionic pair of mono- and bivalent cations in an adequately balanced ratio. To elucidate the possible effects of these ions on the subsequent step of sexual reproduction, i.e., mating activity, the follow-

ing experiments were performed. In view of the fact that the composition of the incubation media may probably have been considerably altered after a culturing period of 15 hrs. (see above), the mating test was carried out with washed gametes suspended in a fresh solution of given composition. For this purpose, growing cells of each mating type were washed and suspended in a solution containing KCl (5 mM.) and MgCl_2 (5/8 mM.), and incubated in the light for 15 hrs. After this treatment, the harvested cells were found to be sexually fully active. The gametes of each mating type were washed twice with distilled water and distributed into fresh salt solutions of 17 different kinds (Table 2). After 3 hr.-incubation in the light, the cells of the complementary mating types in each set of salt solution were mixed and kept in the dark. Counts were made for the mating pairs formed in 2.5 hrs. As shown in Table 2, the mating activity was found to be the highest in a solution containing both KCl and MgCl_2 . The differences in effects of promoting and suppressing elements are much more clear-cut than those in the previous case of the induction of sexuality (Table 1). Here, the presence of bivalent cations with a single exception of Ba^{++} , promoted the mating, while monovalent cations such as K^+ , Na^+ , or NH_4^+ markedly suppressed the process. The suppressing effect of N-substances such as NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , $(\text{NH}_4)_2\text{HPO}_4$, $\text{Ca}(\text{NO}_3)_2$ and KNO_3 are also clearly demonstrated.

To recapitulate, Mg^{++} promotes both sexual differentiation and mating reaction, while Ba^{++} , Li^+ , NH_4^+ and NO_3^- inhibits both processes. Such factors as Na^+ and K^+ promote only the induction, while Ca^{++} only the mating process.

Table 2. Mating frequencies (%) of the sexually functional cells in inorganic salt solutions (5 mM. each)*

KCl and MgCl_2^{**}	72.5	Na_2SO_4	1.5
Distilled water	35.2	$(\text{NH}_4)_2\text{SO}_4$	0.5
LiCl	0.3	MgSO_4	66.3
KCl	0.6	NH_4NO_3	0.8
NaCl	1.5	$\text{Ca}(\text{NO}_3)_2$	26.8
MgC	34.9	KNO_3	0.3
CaCl_2	63.8	K_2HPO_4	0.5
BaCl_2	0.6	Na_2HPO_4	0.8
NH_4Cl	1.4	$(\text{NH}_4)_2\text{HPO}_4$	0.8

* Sexually functional cells of each mating-type were separately cultured in each solution, then mixed together and kept in the dark for further 2.5 hrs.

** KCl 5 mM. and MgCl_2 5/8 mM.

Discussion and General Conclusion

One of the most clear facts reported in this study is the depression of sexuality in *C. moewuii* var. *rotunda* caused by the supply of assimilable N in the medium. The mating process *per se* is also found to be strongly affected by the same factor. Nutritional balance seems to make one of the possible mechanisms causing dedifferentiation of the gametic activity in the presence of assimilable N. In fact, when N was assimilated by the N-starved cells, synthesis of material for vegetative growth must recur, thus providing a physiological state unsuitable for mating. Sager and Granick⁷⁾ reported in their attempts to compare C/N ratio of the gametes with that of the vegetative cells in *C. reinhardi*, whose sexuality was also shown to be dependent on

the concentration of N in the medium, that the ratio did not correlate with the sexual activity of cells. They concluded that induction of sexuality was controlled not by the level of total N, but by the concentration of some specific N fraction or compounds in the cell.

According to Syrett¹⁴), when $(\text{NH}_4)_2\text{SO}_4$ was added to the culture of N-starved cells of *Chlorella vulgaris*, 75~80% of the added ammonium was assimilated, in the first 30 min., into the fraction of soluble N, and also into insoluble N, presumably chiefly protein. Although sexuality has not been reported in *Chlorella*, the circumstance concerning such assimilating processes must also be the case in *Chlamydomonas*. The question remains open for future investigation whether such sequence of events towards synthesis of cellular N-material prevents the formation (or induces the exhaustion) of sexual substances in the cell, and thus suppresses (or dedifferentiates) sexuality. Certain types of sexual substance have been demonstrated in *Chlamydomonas*^{1,9,15}).

On the other hand, it was also unambiguous that cells cultured in the ionic unbalanced medium could not perform their sexual reproduction. A combination of mono- and bivalent cations as K/Ca or K/Mg provided medium suitable for the occurrence of sexuality. The promoting effect of Ca^{++} or Mg^{++} on the mating reaction observed in the present organism was in agreement with that described by Lewin¹⁶) in the type strain of *C. moewusii*. There have been numerous works reporting that the importance of Ca^{++} for the fertilization in various organisms¹⁷⁻¹⁹). Inasmuch as Ca^{++} or Mg^{++} is known to induce gelation at cell cortex¹²), such a physical alteration may take place, in the presence of these ions, at the flagella surface of *Chlamydomonas*; this will favor the contact and adhesion of cells at the tips of flagella in the mating. In contrast to the above mentioned activity of the bivalent cation, all the monovalent cations inhibit the mating reaction; NH_4^+ may not be an exception. However, the fact observed in the dedifferentiation-experiment would not have been due to the difference in the ionic activities between NH_4^+ and Na^+ , but due to the nutritional one. For, those media seem to be well balanced in the rest of other factors.

As observed in the experiments for induction of sexuality, this process preferred K^+ or Na^+ to Ca^{++} , i.e. the two processes in the sexual reproduction quite differed from each other in their ionic preference. Nevertheless, it was emphasized that none of those ions tested in the present study could hold the full sexual activity, and also that none of them was indispensable for the sexual reproduction; a physiological balance in the culture medium produced by the ionic interaction was an essential factor for the performance of sexuality.

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Summary

Induction of sexuality in *Chlamydomonas moewusii* var. *rotunda*, which was used as *C. sp.* 24 in the previous experiment, was dependent on the concentration of assimilable N in the medium (Tsubo, 1956²). The sexual activity of cells dedifferentiated also in the presence of assimilable N. Since it was supposed that the inhibition of mating reaction by N would be due not only to the nutritional, but also to the ionic interference, the experiments were performed to see the effect of each ion

used in the culture medium on the sexual reproduction of this alga. Bivalent cations such as Ca^{++} and Mg^{++} promoted, but monovalent cations such as K^+ or Na^+ inhibited the mating reaction. On the other hand, K^+ , Na^+ and Mg^{++} , but not Ca^{++} promoted the preceding step, induction of sexuality. NH_4^+ and NO_3^- inhibited both of these processes. Furthermore, it was noticed that none of these ions was indispensable, but a physiological balance in the medium produced by the ionic interference, e.g. with K^+ and Ca^{++} , or K^+ and Mg^{++} , was important for the accomplishment of sexual reproduction.

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摘 要

坪 由 広: クラミドモナスの生殖に対する培地のイオン組成の影響について

Chlamydomonas moewusii Gerloff var. *rotunda* Tsubo において、配偶子形成が培地中の N 源の濃度に依存していることを、著者はすでに報告した。一方、N 源の存在するときには配偶子の接合能力がおさえられ、配偶子はふたたび栄養細胞になるものと思われた。接合の阻害機構としては、N 源のとりこみによる栄養的阻害が考えられるが、他方、接合現象が鞭毛の接触により始まるので、この表面現象に対する物質のイオンとしての阻害作用も考えられねばならない。そこで、本研究ではこの藻の有性生殖に対する培地のそれぞれのイオンの作用についてしらべてみた。その結果、 Ca^{++} や Mg^{++} のような 2 価の陽イオンは接合を促進し、 K^+ や Na^+ のような 1 価のものはこれを阻止した。逆に K^+ , Na^+ , Mg^{++} は前段階すなわち配偶子形成をうながし、これに対し Ca^{++} は逆の立場にあると考えられた。 NH_4^+ および NO_3^- は、ともに上記 2 つの生殖段階のいずれをも阻止した。さらに、本実験において試みられたイオンの中には、それらのうちどれかが生殖にとって必要不可欠であるというものはないが、この現象が遂行されるためには、培地中のイオン相互作用、たとえば K^+ と Ca^{++} 、あるいは K^+ と Mg^{++} のような組み合わせによって生じる生理的平衡が重要な因子であることがわかった。(神戸大学理学部生物学教室)