*CELLULAR DIFFERENTIATION IN VOLVOX**

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Microorganisms have become important research materials in the study of differentiation at all levels of organization from the molecular to the cellular, but there remains a wealth of species whose potential has not as yet been realized due to problems of isolation, cultivation, or manipulation of the various phases of the life cycle. In this latter group the green alga *Volvox* has for many years been recognized as having aspects of organization and development that would make it a prime species for investigation of differentiation at the cellular level. The *Volvox* individual is a spheroid in which the biflagellate cells are arranged in a single peripheral layer. Two types of cells are always present: somatic cells which characteristically make up the bulk of the organism; and reproductive cells which occur in small numbers and which may be differentiated as gonidia, i.e., asexual cells capable of reproducing new individuals without fertilization, or sexual cells capable of becoming eggs, or of forming packets of sperm cells. It is of special interest that many of the species of *Volvox* were delimited by such early workers as Powers[1] and Shaw[2] using as taxonomic criteria the type and degree of differentiation, and the time at which such differentiation occurred during the development of the young individuals.

The pioneering work by Darden[3] on *Volvox aureus* showed the possibility of studying the control of the reproductive cells in cultured material where growth and development could be examined in detail. Prior to Darden's work, *Volvox* had been grown successfully in both bacterized and axenic culture, but there were no reports in the literature of the control of the sexual phases in such cultures. Using a defined medium designed for *Volvox globator* by Provasoli and Pintner,[4] who apparently achieved excellent growth but recorded no sexual phases, Darden reported for the first time the growth and reproduction, both asexual and sexual, of a *Volvox* species in controlled culture. Darden's M-5 strain of *V. aureus* had been isolated from a natural population and after being freed of bacteria by repeated washing of young individuals, the strain was grown axenically in the Provasoli and Pintner culture medium. After approximately 14 days, male individuals producing sperm packets were evident in the population. Upon release, the sperm packets sought out and penetrated young individuals apparently containing asexual reproductive cells (gonidia). Under these circumstances the gonidia acted as eggs, were fertilized by the sperm, and after a period of enlargement became transformed into typical orange-colored dormant zygotes. The failure to re-establish asexual populations by the transfer of small amounts of inoculum from sexual cultures prompted Darden to investigate the phenomenon of sexual induction more fully and thus led to his discovery of the presence in the filtrate of sexual cultures of a substance (or substances) which in small amounts would induce the formation of males in cultures that otherwise would have produced only asexual individuals.

Since 1965 there has been a concentrated effort by the writer, his students Dr. Gary D. Kochert and Mr. Michael McCracken, and his research assistant, Mr. Richard W. Nelson, to collect, isolate, and evoke the sexual phase in as many as possible of the 18 species recognized by Smith[5] in his monograph on the genus. Thirteen species are now in culture and have been positively identified. In addition, several other species are represented among a large number of clonal populations of the section *Euvolvox*, but positive identification awaits a prerequisite detailed study of the members of this section. Sexual phases have been observed in all species in culture. Table 1 indicates the characteristics of the various strains[6] as to sexual type.

Induction systems have been demonstrated in eight of the strains listed in Table 1. With the exception of *V. aureus*, all systems of induction depend on the production of an active substance by the male strain.[7] Darden[3] believes that the active principle in *V. aureus* is secreted by asexual individuals as the culture ages, but Kochert[8] and the writer, using different varieties of *Volvox carteri*, have evidence which indicates that in their material the active principle is produced by the male individuals. The effect of the male filtrate in the various strains is as follows:

(a) *Volvox aureus* (M-5 strain): This is the only homothallic species in which an induction system has been positively identified. The filtrate of a sexual culture causes production of males in cultures that otherwise would have produced only asexual individuals at that time (Darden[3]).

(b) *Volvox aureus* (65-98 strain): Males have not been observed in this strain; but as cultures age, gonidia enlarge and, without dividing, are transformed into orange-colored parthenospores. The filtrate from such cultures will induce the formation of parthenospores in the 65-98 strain and of male individuals in the M-5 strain. The filtrate from the M-5 strain will induce parthenospores in the 65-98 strain. Darden[9] reported the induction of males in the M-5 strain using the filtrate of another parthenosporic strain.

(c) *Volvox carteri* var. *weismannia* (NB3 male and NB7 female): Kochert[8] has shown that the production by the female strain of individuals bearing eggs is dependent on the presence of male individuals or of the filtrate of sexual males. The male strain cannot be self-induced by male filtrate in the strains studied by Kochert.

(d) *Volvox carteri* var. *weismannia* (Waterford 4 male and Waterford 3 female strains): In these strains from Australia, the male filtrate is active only in inducing eggs in the female strain. The Australian male will not induce egg formation in the Nebraskan females, nor will the Nebraskan male induce eggs in the Australian female of the same species. The time at which the females are sensitive to the male filtrate is different in the two strains, and this will be discussed later.

(e) *Volvox carteri* var. *nagariensis* (HK 9 male and HK 10 female): These strains from Japan are like the preceding two sets of *carteri* strains in that the female strain will form eggs only in the presence of the male individuals or male filtrate. The filtrate from the Japanese male will not induce eggs in the female strains of the Australian and Nebraskan strains, nor is the Japanese female

TABLE 1. *Sexual types of Volvox species in culture.*

Name of species	Strain designation	Origin	Isolator	Homothallic	Heterothallic	Monoecious	Dioecious	Parthenosporic
V. africanus	Mo	Missouri	Tindall	X			X	
V. africanus	Darra	Australia	Starr		X		X	
V. aureus	M-5	Michigan	Darden	X			X	
V. aureus	65-98	Mississippi	Starr					X
V. barberi	IU 804	California	Stein	X		X		
V. carteri var. *nagariensis*	HK	Japan	Starr		X		X	
V. carteri var. *weismannia*	NB	Nebraska	Kochert		X		X	
V. carteri var. *weismannia*	Wf	Australia	Starr		X		X	
V. dissipatrix	Marburg	Australia	Starr	X		X		
V. gigas	K25	South Africa	Starr		X		X	
V. globator	IU 955	Massachusetts	Goldstein	X		X		
V. obversus	Wd	Australia	Starr		X		X	
V. perglobator	HP	Indiana	Starr and McCracken		X		X	
V. powersii	17-10	Nebraska	Kochert	X			X	
V. rousseletii	K32	South Africa	Starr		X		X	
V. spermatosphaera	17-14-4	Nebraska	Kochert and Starr	X			X	
V. spermatosphaera (small form)	H	Missouri	Kochert and Starr	X			X	
V. tertius	IU 132	England	George	X			X	
V. sp. (*Euvolvox*)	62-22	Mexico	Starr	X		X	Males	

Homothallic: sexual reproduction occurring within a clonal population.
Heterothallic: sexual reproduction occurring only between two different clonal populations.
Monoecious: both eggs and sperm formed in the same individual.
Dioecious: eggs and sperm formed in separate individuals.
Parthenosporic: forming resistant spores without fertilization.

strain sensitive to the male filtrates of these other *carteri* strains. The filtrate from the Japanese males can also be used as a self-inducer; thus asexual individuals of the male strain can be induced to form males rather than asexual individuals by the addition of male filtrate to the medium at the proper time. Self-induction is not possible in the Australian or the Nebraskan male strains.

(f) *Volvox rousseletii* (K32 male and K32 female strains): This species, known only from South Africa, shows induction of both males and females in the respective strains by the male filtrate, but, in contrast to the Japanese strains of *V. carteri*, the females may also form eggs under certain environmental conditions even without the presence of the male filtrate.

(g) *Volvox obversus* (Wd 7 male and Wd 3 female strains): This species from Australia is similar to the Japanese *carteri* in that both males and females are induced by the male filtrate.

(h) *Volvox gigas* (K25 male and K25 female strains): In this South African species the male filtrate induces sperm production by the male strain. The female strain does not produce special eggs, but, as in *V. aureus*, the asexual reproductive cells may function facultatively as eggs.

The actual time at which the inducer molecules in the male filtrate enter into the metabolic pathways leading to the production and/or development of sexual cells rather than asexual cells is as yet unknown, but the great diversity among the different strains as to when the male and/or female strains can be effectively induced to form sexual individuals indicates that the molecular aspects of the inductive systems also may be diverse.

Darden[3] has shown that as little as a five-minute exposure to the sexual filtrate is effective in inducing the production of males in the M-5 strain of *V. aureus*, provided this is done during a certain critical period in the development of the individual. This critical period in *V. aureus* is thought to be just prior to the inversion of the young individual, a stage which immediately follows the cessation of all cell divisions (Fig. 1, stages 3, 7). In contrast, in the induction of parthenospores using the 65-98 strain of *V. aureus*, the writer has found that it is the very young individual (stages 1, 5), very shortly after its release and before any cleavages have occurred in the gonidia, that must be exposed to the active filtrate (of the M-5 strain or a parthenosporic culture of strain 65-98). When induced, the gonidia in the young individual do not divide but are transformed into large resistant parthenospores similar to dormant zygotes in appearance.

Preliminary experiments by the writer with *Volvox rousseletii* (K32 male strain) have shown that young individuals which have completed inversion and are enlarging preparatory to their release from the enclosing sphere of the parent individual can be induced to become males by the addition of male filtrate to the medium (stages 4, 8). Examination of the young individuals at this stage shows that reproductive cells with male potential are present in all young individuals of the male strain, but unless they are induced to develop by the presence of the active substance in male filtrate, a typical asexual individual develops with gonidia forming the next generation. In noninduced individuals, the potentially male cells develop as typical somatic cells, their identity becoming less and less distinct as the individual enlarges and matures.

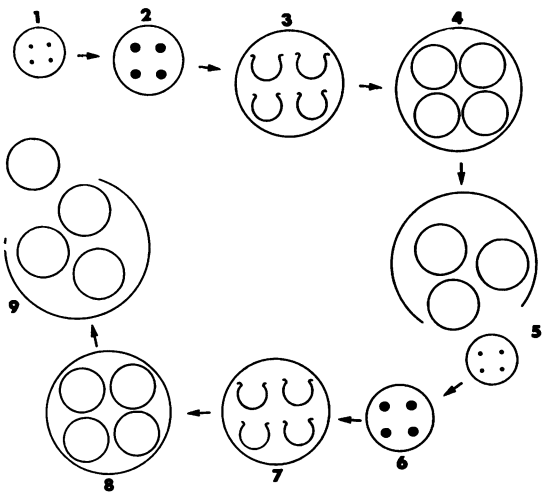

FIG. 1.—Asexual reproduction in *Volvox*. Diagram showing the development of two successive asexual generations in *Volvox*.

The gonidia of a young individual (stage *1*) undergo enlargement and successive divisions (stage *2*) forming new individuals which invert (stage *3*), enlarge (stage *4*), and later are released from the parent individual (stage *5*). The young asexual individuals thus produced contain gonidia (stage *5*) which go through a period of enlargement and successive divisions (stage *6*) to form new individuals which invert (stage *7*), enlarge (stage *8*), and escape from the parent (stage *9*).

This process is repeated forming asexual individuals, unless sexual induction has been initiated at one of the stages illustrated in the drawing. The stage at which exposure to the sexual inducer is effective varies with the species and/or strain under consideration. See the text for details.

It is somewhat surprising to find that induction of the sexual stages may be delayed even farther than in *V. rousseletii*. In sexual strains (K25 male and female strains) of *V. gigas* from South Africa, young individuals are released from the parent individual (stages 5, 9) and to all appearances are asexual with many gonidia in the posterior half of the organism. However, such seemingly asexual individuals in the male clone may be transformed directly into males by exposure to male filtrate. In such males, the asexual reproductive cells divide to form large packets of sperm and, more surprisingly, many of the somatic cells in the posterior half enlarge slightly, divide, and form small packets of sperm. In no other species of *Volvox* do cells once set aside as somatic cells regain the ability to divide mitotically.

In the various *carteri* strains induction is not a matter of the development of potentially sexual cells already present as it is in *V. gigas* and *V. rousseletii*, and as it may well be in *V. aureus*, but rather, the inductive effect of the male filtrate is in the production of the sexual cells (or inhibition of asexual cell production) in the early stages of the development of the individual. Powers,[1] in naming *V. weismannia* (now *V. carteri* var. *weismannia*), chose that name because of the early delimitation of the reproductive cells in the developing individual. Kochert[8] has shown that in this species the first gonidia are delimited by unequal divisions of the first and second tiers of cells at the time the 16-celled stage divides

to form 32 cells; however, when effectively induced by the male filtrate, the first two tiers of cells in the 16-celled embryo divide equally and, as a result of this and subsequent divisions, egg cells are formed. In var. *nagariensis* (HK strains) and var. *weismannia* (Wf strains) induction is possible if the young individuals (stages 1, 5) are allowed to develop in the presence of the male filtrate, during which period the gonidia that will ultimately produce the next generation, be it sexual or asexual, enlarge greatly. The necessary time of exposure to the filtrate is as yet unknown; however, it is evident that exposure after the beginning of the first cleavage is too late to affect the later divisions as to the type of reproductive cell delimited. Kochert,[8] studying var. *weismannia* (NB strains), has shown that the effective period is much earlier than in the above-mentioned strains. In Kochert's strains the gonidia apparently are susceptible only during the early stages of their enlargement before the individual to which they belong has been released from its parent (stages 3–4). Thus, in his strains, the exposure of a recently released individual (stage 1) to male filtrate will produce only asexual individuals in the first generation (stage 5); however, these asexual individuals will produce females in the second generation (stage 9) regardless of whether the development of the second generation takes place in the presence or absence of the male filtrate.

In *Volvox obversus* (Wd strains) both males and females can be induced by the active male filtrate provided there is sufficient exposure to the filtrate during the time of gonidial enlargement. It is necessary to expose the individuals in these strains at an early age (stages 1, 5), as is the case with the Japanese and Australian strains of *V. carteri*.

Work is now in progress[10] on the biological action of the inducer and its chemical nature. Darden[3] considers the active material in *V. aureus* to be proteinaceous because of its heat lability and its inactivation by certain proteolytic enzymes; filtration through Sephadex columns indicates the molecular weight to be in excess of 200,000. Additional unpublished data from Darden's laboratory continue to uphold his view of the proteinaceous composition of the active substance. Kochert[8] considers the active substance in *V. carteri* var. *weismannia* (NB strain) to be proteinaceous also, due to its heat lability and decomposition by proteolytic enzymes; but, in this strain, Sephadex filtration yielded two active fractions, the smaller with a possible molecular weight in the range of 10,000–15,000. The writer's investigations with *V. carteri* var. *nagariensis* (HK strain) have not been in progress long enough to be certain of the size and class of the compound, but dialysis and heat experiments indicate that in this variety the active inducer may well be proteinaceous also. As yet, little is known of the inducers in the other strains. Whatever the nature of the inducer may be, it is obvious that it is effective in low concentrations and appears to be species- or, in some instances, strain-specific. Darden reports that some batches of male filtrate in *V. aureus* give a small degree of induction in concentrations of 10^{-5}. Kochert reports similar activity in filtrates of *V. carteri* var. *weismannia*. The writer has demonstrated 100 per cent induction in 10^{-6} concentrations of the male filtrate, with appreciable activity down to 2×10^{-7}.

Many experimental techniques must still be perfected in studying each

particular strain of *Volvox*, but the results at hand show that the unique features of organization and development in *Volvox* which have interested developmental biologists for so long can now be investigated under the controlled conditions necessary for an understanding of the biological and chemical nature of the differentiation process.

Summary.—The axenic culture in a defined medium of multiple species of *Volvox*, the growth and reproduction of which can be controlled, provides material for a continuing study of the chemical basis of differentiation in a multicellular organism with only two cell types, somatic and reproductive. Induction of sexual reproductive cells through the use of filtrates from sexual male cultures has been demonstrated in eight different strains. The type of response by the male or female strains and the time during the developmental cycle at which the inducing substance is active differ among the strains.

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[5] Smith, G. M., *Trans. Am. Microscop. Soc.*, **53**, 265 (1944).

[6] Only representative strains are listed in Table 1. Many species are represented by several strains of diverse geographical origin and are maintained in the writer's laboratory.

[7] In all strains the production of male colonies is a spontaneous event that can be controlled by careful regulation of environmental factors such as media, temperature, and light. The various strains respond differently, and so those environmental conditions necessary to induce or to inhibit the production of male colonies must be determined for each individual strain.

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[10] W. H. Darden, Jr., University of Alabama, and G. D. Kochert, University of Georgia, are continuing their respective investigations with *V. aureus* and *V. carteri* var. *weismannia*. M. D. McCracken, Indiana University, is studying the system in *V. rousseletii*, and the writer is investigating those in *V. carteri* var. *nagariensis* and other strains.