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L-Glutamic acid as a mediator of sexual morphogenesis in *Volvox capensis*

(sex hormone/ecology)

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ABSTRACT **In *Volvox capensis* the development of sexual individuals is in response to low concentrations (68 nM) of L-glutamic acid rather than to such species-specific glycoproteins as have been isolated in *Volvox carteri* or are believed to exist in a number of other species. *V. capensis* grows equally as well in light and in darkness in a medium supplemented with sodium acetate; however, L-glutamic acid is active as an inducer of the sexual form only in populations grown in the light. The site of action of L-glutamic acid and its biochemical role in the sexual response are unknown. Attempts to induce the sexual response by using the other L-amino acids, various analogs of glutamic acid, compounds of similar structure (e.g., γ-aminobutyric acid), and intermediates of biochemical pathways known to involve L-glutamic acid (e.g., α-ketoglutarate or pyroglutamic acid) have been unsuccessful. L-Glutamic acid is produced by *V. capensis* as a natural product of the digestion of the glycoproteinaceous parental matrix at the time young spheroids escape. As a population increases, so does the level of L-glutamic acid produced at each succeeding generation until the threshold of sensitivity is reached and the induction of sexual forms is effected. This serves as a mechanism for ensuring the production of sexual spheroids and their zygotes, the only phase in the life cycle resistant to drying. Thus, *Volvox* is especially adapted to an existence in ephemeral pools of water resulting from seasonal rains.**

During the past 15 years the control of cellular differentiation has been examined in a number of different species of *Volvox*: *V. aureus* (1), *V. carteri* f. *weismannia* (2, 3), *V. carteri* f. *nagariensis* (4, 5), *V. gigas* (6), *V. rousseletii* (7), and *V. dissipatrix* (8). Asexual spheroids are formed in successive generations, but, if a species-specific sex hormone is present during development of the embryos, sexual spheroids will result. The hormone is produced only by male spheroids, except in the single species, *V. dissipatrix*, where it is produced by both sexes. In *V. carteri*, from which the hormone has been isolated and characterized, it is a large glycoprotein of M_r 25,000–30,000 (9, 10). Even in other species the hormones appear to be large glycoproteins, but none has been isolated yet. Until recently the investigations have been confined to dioecious species (i.e., those having separate male and female individuals), but Palmer (11) reported details of an induction system in two monoecious species of *Volvox* (i.e., having both eggs and sperm in the same spheroid). Palmer showed that the M_r of the hormone, determined by gel filtration, was possibly less than 1400, but the strains with which he worked did not lend themselves to production of the hormone in quantities large enough for extraction and characterization. This prompted our re-examination of a large number of monoecious isolates of *Volvox* that had been isolated from all over the world during the past 13 years. It is with one of these, the K37 strain of *V. capensis* Rich et Pocock from South Africa, that this report is concerned.

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MATERIALS AND METHODS

The K37 strain of *V. capensis* was isolated from soil samples (12) collected near Kimberley, South Africa. Cultures were grown axenically in *Volvox* medium (4) adjusted to pH 7.5 and supplemented with thiamine and sodium acetate at 0.1 mg per 100 ml and 0.1 g per 100 ml, respectively. With the addition of acetate to the medium, it was possible to maintain the stocks in total darkness, where they would remain in the asexual state. The alga grew well at 25°C, having a generation time of approximately 36 hr. At 30°C the generation time approximated 24 hr. For experimental purposes, lighting was provided by General Electric Power Groove fluorescent tubes at an intensity of 6000–7000 lux.

The bioassay of the sexual inducer involved making a serial dilution of the material to be tested in 10-ml blanks of *Volvox* thiamine/acetate medium. The blanks were made from sterile *Volvox* medium (pH 7.5) to which were added sterile thiamine solution (0.333 mg/ml) and sterile sodium acetate solution (0.1 g/ml); to each 100 ml of *Volvox* medium, 1 ml of each sterile stock was added. Each assay tube was inoculated with a single parent spheroid containing 10–15 embryos in the predent stage of development. The tubes were illuminated on a 16-hr light/8-hr dark cycle at an intensity of approximately 6500 lux at 27°C. After 48 hr the progeny of the young embryos in the inoculum were examined under the dissecting microscope. In those dilution tubes in which the sexual inducer was not limiting, approximately 100% of the progeny would be sexual; at that dilution in which the inducer became limiting, less than 100% induction could be observed. Further dilutions showed no induction.

RESULTS AND DISCUSSION

Although the structure of the spheroid of *V. capensis* and its asexual and sexual development have been described in great detail by Pocock (13), certain aspects are of special importance in the consideration of the control of sexual differentiation mediated by a substance dissolved in the surrounding medium. The mature spheroid is composed of a single layer of approximately 10,000 small biflagellated somatic cells plus a small number of reproductive cells. Newly released spheroids are composed of three sizes of cells. The smallest and most numerous become the somatic cells. Scattered among the somatic cells are 20–60 intermediate-sized cells, and, depending on the culture conditions, from as few as 4 (in depressed populations) to as many as 25 (in vigorous populations) much larger cells.

In asexually developing spheroids, the intermediate cells can be observed only for a short period, after which they become indistinguishable in appearance and function from enlarging somatic cells. In contrast, the 4–25 large cells function as gonidia in asexual reproduction. Successive cleavages in an embryo developing from a gonidium are separated in time by approximately 2 hr, during which the cells increase in size. Shortly

before the last cellular division in the embryo, the large cells, which will become gonidia, become evident. With the completion of the cleavage process, the embryo expands unevenly and its cellular surface becomes highly dented. The dent period is transient and is soon followed by inversion of the embryo, during which the embryo turns inside out. Usually within 6 hr after inversion, the enlarging embryos are released individually from the parental spheroid by digestion of the glycoproteinaceous matrix of the parental spheroid in the region of each enlarging embryo.

Sexual spheroids are similar to asexual ones in their size and in the number of somatic cells, but they differ in the number and nature of the reproductive cells. Mature sexual spheroids of *V. capensis* are monoecious, with 10–15 yellowish sperm spheroids and 60–120 dark green eggs scattered among the somatic cells in the posterior two-thirds of the spheroid.

As mentioned above, shortly after the escape of a fully developed embryo from its parental spheroid, three different-sized cells are apparent. Observations of the sexual mode of development reveal that the small cells remain somatic in function. The intermediate-sized cells, which become indistinguishable from somatic cells in the asexual spheroid, become dark green in pigmentation as they enlarge and develop into eggs. The eggs are flask-shaped with the enlarged portion of the cell projecting inward from the periphery. The large cells, functioning as gonidia in the asexual mode of development, divide and invert to form compressed spheroids of 512 flagellated sperm cells each.

Before the eggs have completely matured, sperm spheroids begin to escape individually to the outside of the sexual spheroid and swim actively before attaching to the same or another spheroid, commonly in the posterior region. Individual sperm exit through a common pore and penetrate the matrix of the attached sexual spheroid. Fertilization is assumed to occur, resulting in the production of orange-colored spiny zygotes: however, the actual fusion of a sperm with an egg has not been observed in this species. Zygotes are passively released from the sexual spheroid as it ages and gradually disintegrates.

Mixed individuals, in which both sexual and asexual reproductive cells are present, were occasionally observed. In such spheroids the sexual and asexual reproductive cells are not randomly scattered, but each type is confined to a particular section of the spheroid. Thus, a mixed spheroid may have a sexual anterior and an asexual posterior, or the divisions may be along the longitudinal axis.

In other species in which induction systems have been studied, the apparent "spontaneous" development of a large sexual population within a single generation in a culture vessel is usually predicated on the unnoticed appearance in the previous generation of a few males that produce enough sex hormone to "turn on" the developing embryos of the next generation, thus producing what appears to be a spontaneous effect. In the K37 strain of *V. capensis*, spontaneously developing sexual populations could be observed after a population increased in numbers, but this occurred without any sign of any previous sexual individuals. In *V. capensis*, unlike *V. carteri* in which the first sexual males form and then dissociate into component cells and sperm packets, a sexual spheroid with both sperm and eggs becomes even more evident as the fertilized eggs mature as orange-colored zygotes. Yet single asexual spheroids of *V. capensis* inoculated into tubes of medium produced sexual populations within a predictable number of generations, but a sexual spheroid was never observed as the necessary precursor of the reaction. Media of lower concentration did not speed the appearance of the sexual reaction, thus indicating that depletion of nitrogen or some other regular component of the medium was not responsible for the onset of the sexual process, as has been reported for some other algae (14). In contrast, a 1:1000 dilution of fluid from a spontaneously sexual population was effective in eliciting the sexual response, reinforcing the idea that a sexual inducer was present. In the absence of a "first" sexual individual to secrete the sexual inducer, it was hypothesized that some substance was being produced during the growth of the population which eventually reached a level of concentration such that, during the development of the embryos at that time, the sexual pattern of development was induced.

The time of the spontaneous appearance of sexual spheroids could be predicted as a function of the size of the original inoculum and the volume of the medium used. When a single parental spheroid containing approximately 15 developing embryos was inoculated into a tube containing 10 ml of *Volvox* thiamine/acetate medium, the first generation of 15 asexual spheroids produced in the second generation 225 asexual spheroids, and from these 225 spheroids a third generation of >3000 spheroids was formed, the majority of which were sexual. However, sexual spheroids appeared in the second generation rather than the third if the original inoculum was increased (five parental spheroids in 10 ml of medium) or if the volume of the medium was decreased (one parental spheroid in 2 ml of medium). The parental matrix was considered as a prime candidate for the source of the sexual inducer, inasmuch as the release of the offspring is through the digestion of this matrix, a glycoprotein. The medium from old populations of other species of *Volvox* was tested for inducing activity by making a dilution series in 10-ml blanks of *Volvox* thiamine/acetate medium and inoculating with single parental spheroids of *V. capensis*. Sexual spheroids appeared in the second generation, indicating that the same inducer, or one with similar activity, was present. Control tubes without the old medium produced asexual populations in the second generation. The sexual inducer to which the K37 *V. capensis* would respond was not active on any of the species from which the old populations had been derived.

L. Jaenicke (Institut für Biochemie, Köln) had shown earlier during a stay in our laboratory that the releasing factor from *V. carteri* f. *nagariensis* was an enzyme that would digest the matrices of other species of *Volvox* as well. Furthermore, a much earlier observation by Kochert (2) showed that Pronase (Sigma protease VI) could be used to digest the matrix of *V. carteri* f. *weismannia*. Because naturally occurring enzyme preparations are not available, asexual spheroids of *V. capensis* were digested with Pronase (1 mg/ml, 24 hr, 37°C, pH 8), and, when assayed, the digest was found to have activity at a 1:10,000 dilution.

Other proteins subjected to digestion by Pronase showed activity to varying degrees. Mucin, ovalbumin, hemoglobin, casein, zein, gluten, and enzymatic hydrolysate of casein all surprisingly gave good activity after digestion. And when enzymatic hydrolysate of casein (100 mg/ml) was assayed even prior to digestion, high activity at a $1:10^5$ or $1:10^6$ dilution in the assay could be achieved, the variation depending on the particular source of the hydrolysate. Thus, it became possible to devote our extraction efforts to an easily obtainable source of the inducer rather than having to grow large quantities of *Volvox* as had been necessary in the isolation of the inducers from *V. carteri* (9, 10).

When run through gel filtration columns (Bio-Gel P-2 with exclusion limit of 2000 M_r; ammonium bicarbonate buffer at 0.1 M and pH 7), the peak of activity was eluted in the fractions immediately after the peak of vitamin B_{12} used as a marker (M_r 1355). This indicated a molecular size of about 10 amino acids, a figure in the range of M_r 1400 for the inducer described by

Palmer. Subsequent experimentation was designed for the extraction of a small peptide that, at the time, was believed to be remarkable in its ubiquitous presence as a unique sequence in various proteins, or as a smaller active site of several amino acids in combination with others to give a certain effective size. Resistance to Pronase digestion, to heat, and to acid denaturation only added to the mystery of the nature of such a ubiquitous peptide. Active fractions from the P-2 column, chromatographed at first on thin-layer silica gel plates and later on filter paper, (butanol/acetic acid/H_2O), showed four spots, the second one from the origin having all the activity. Subsequent adsorption on DEAE-cellulose and elution with 0.1 M NaCl in buffer, followed by gel filtration a second time, yielded active fractions that showed only two spots, one of which contained all the activity. High voltage electrophoresis was used to separate the two components in preparation for amino acid analysis. In contrast to the expected small peptide, the analysis showed the presence of glutamic acid with a very minor contamination of other amino acids. When assays of commercially prepared glutamic acid (D-glutamic acid and L-glutamic acid from Sigma) were assayed, the L-glutamic acid alone showed activity. At 680 nM, there was 100% induction in the assay; at 68 nM, induction was higher than 80%. No induction was seen at 6.8 nM. Similar results had been obtained with the purified inducer from the casein hydrolysate. Furthermore, the assay of fractions from L-glutamic acid run on a Bio-Gel P-2 column showed activity in the same fractions as the inducer from casein hydrolysate, and chromatography of the fractions showed the L-glutamic acid to have the same R_F value. The active factors isolated from K37 *V. capensis*, ovalbumin, and casein showed identical mobility in high-voltage electrophoresis, indicating the same composition, the identity of which could be only L-glutamic acid.

The bioassay of all other L-amino acids, including glutamine, has yielded negative results except in those instances in which the amino acid preparation is known or thought to be contaminated with L-glutamic acid. For example, L-glutamine (Sigma) activity in the bioassay at a level of less than 1% that of L-glutamic acid was expected because of the presence of L-glutamic acid as a contaminant at the 0.5% level. Various analogues and intermediates from metabolic pathways, closely or more remotely involving glutamate, have been assayed, but as yet nothing has been found with specific activity greater than 1% of that of L-glutamic acid (See Table 1).

Table 1. Compounds tested for activity in inducing sexual morphogenesis in *V. capensis*

N-Acetyl-DL-glutamic acid
N-Acetyl-L-glutamic acid
λ-Amino-*n*-butryic acid
ϵ-Amino-*n*-caproic acid
δ-Amino-*n*-valeric acid
D-Glutamic acid
L-Glutamic acid-λ-monohydroxymate
λ-L-Glutamyl-L-glutamic acid
λ-L-Glutamyl-L-glutamine
λ-L-Glutamyl-L-leucine
λ-L-Glutamyl-L-valine
Glutaric acid
Glutathione
λ-Hydroxybutryic acid
α-Ketoglutaric acid
β-Methyl-DL-aspartic acid
DL-α-Methylglutamic acid
N-Methyl-DL-glutamic acid
L-Pyroglutamic acid

No compound showed activity unless the concentration was at least 100 times that of L-glutamic acid, which is active at 10 μg/liter.

Early in the investigation, it had been noted that light was necessary if the sexual inducer were to be effective. The K37 strain of *V. capensis* is unusual among *Volvox* strains in being able to utilize acetate as a carbon source in the dark. In fact, although *V. capensis* is capable of autotropic growth through photosynthesis, the growth rate in the light is stimulated noticeably with the addition of sodium acetate at 0.1 g per 100 ml. Growth with acetate in the dark and in the light appears to be equal, the generation time reflecting the temperature at which the organism is grown.

To be effective as a sexual inducer, the glutamate must be added early in the development of the embryos, usually no later than the 64-cell stage. Illumination of these embryos (within their parents) may be delayed until just prior to the onset of inversion, a stage recognized by the dented appearance of the surface of the embryo. A period of 10–15 hr of light at this time is necessary for successful induction (15). Short exposures of light (1 hr or less) at various developmental stages have proved ineffective.

Like many other algae, *Volvox* is commonly found in small pools of water, ephemeral in nature, having been formed by the collection of rainwater in depressions in uncultivated land. In the strategy of life, it is of prime importance to the continued existence of such living creatures that resistant cells always be formed that can survive when the pools dry. This has been dramatically described by Powers (16) who wrote that "in the full blaze of Nebraska sunlight, *Volvox* is able to appear, multiply, and riot in sexual reproduction in pools of rainwater of scarcely a fortnight's duration." The zygotes resulting from this "riot in sexual reproduction" are the resistant phase; the sexual hormones (in *V. carteri* and other species) and L-glutamic acid (in *V. capensis*) are the chemical signals inducing the riot.

In those green algae in which the gametes needed for the sexual act are either transformed vegetative cells or the immediate products of vegetative cells, the signal for the onset of sexual reproduction is correlated often with depletion of nitrogen in the medium, but in *Volvox* this has been superseded by the evolution of a system that ensures that sexual reproduction, and the subsequent formation of resistant zygotes, is induced at the time when conditions are optimal for growth. This is important when one considers that sexual reproduction in *Volvox* requires the production of a special sexual individual in which the gametes are formed. On a cell number basis, this involves the production of 5,000–25,000 more cells from each asexual reproductive cell present in the population at the time the inductive process is put into operation. Under less than optimal conditions of growth, the sexual spheroids would be decreased in size and vitality and would lessen the number of resistant zygotes produced.

The role of L-glutamic acid in the control of sexual morphogenesis in *V. capensis* is surprising in view of its importance and presence as a metabolite in many biochemical pathways in the growth and maintenance of cells. At the low concentration at which it is effective, it is reasonable to believe that in this instance the glutamic acid is acting as a messenger rather than as a metabolite, but as yet the site of action is unknown.

Kirk and Kirk (17) have shown that in *V. carteri* only arginine is actively transported across the cell membrane; however, the application of these results to *V. capensis* may be questionable on several bases. *V. carteri* is not induced by L-glutamic acid, and, in addition, the work on *V. carteri* utilizing labeled amino acids at 2.3 nM did not take into consideration the possibility that the medium in which the inoculum had been grown would have various amino acids at concentrations higher than

10 nM because of the proteolysis of the parental matrices during the several generations of growth that had occurred in the inoculum before its use.

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