

# Differentiation in *Volvox carteri*: Study of Pattern Variation of Reproductive Cells

Rainer Gilles and Lothar Jaenicke \*

Institut für Biochemie, Universität zu Köln, An der Bottmühle 2, D-5000 Köln 1, Bundesrepublik Deutschland

Z. Naturforsch. 37 c, 1023–1030 (1982); received June 1, 1982

*Volvox carteri*, Differentiation, Embryogenesis, Cell Lineages, Pattern Formation

Asexual spheroids of the multicellular green alga *Volvox* are composed of two types of cells: non-flagellated reproductive gonidia and *Chlamydomonas*-like flagellated somatic cells. They are committed by a differentiating cleavage during embryogenesis. The gonidia of the adult spheroids form a symmetrical pattern consisting of four layers of four gonidia each; their position is established already in the embryos by the gonidial initials. Whereas, generally, the 16-gonidia pattern is assumed to be the basic one, most of the spheroids have fewer gonidia (down to 8). The nine possible gonidial patterns (8 to 16 gonidia) are described and correlated to the gonidial stem cells which have been differentiated. Defects in gonidial pattern are of particular interest, since any model of differentiation has to explain not only the basic pattern formed, but also its systematic variations. Our study shows that the pattern reduction is by no means random, but governed by an intrinsic mechanism which shifts the first unequal cleavage from the 32-celled stage to the 16-celled stage. All the patterns formed can be deduced from cleavage pathways involving non-synchronous differentiation of the stem cells. Thus, pattern formation can be correlated to timing and spacing signals regulating events during embryogenesis.

For as long as scientists have tried to approach differentiation processes, they have recognized the green flagellated composite alga *Volvox* as a promising object. Nearly 100 years ago A. Weismann postulated his "germ-plasma"-theory to explain the differentiation of germ and soma cell lines [1]. The species *Volvox carteri* f. *weismannia* was named in honour of him; today the closely related *Volvox carteri* f. *nagariensis* is the best examined species of the genus [2–4].

In the course of phylogenesis *Volvox carteri* has acquired in addition to multicellularity also some attributes otherwise found only in higher organized forms of life. During embryogenesis 11–12 synchronous cleavages are carried out, to give rise to 2000–4000 somatic cells and 8–16 reproductive cells, the gonidia. The latter differentiate by an unequal cleavage early in embryogenesis. After the cleavages the embryo is inside out with respect to the adult configuration. By a complicated but well studied chain of rearrangements, called inversion, the daughter spheroid is produced which consists of a single peripheral layer of somatic cells enclosing the gonidia, embedded in a mucilaginous matrix

[5, 6]. The young spheroids hatch by an enzymatic degradation of the somatic sheath of the old spheroid, which eventually dies [7].

The one step differentiation of the two types of cells – flagellated somatic cells and non-flagellated gonidia – is a simple, but nevertheless complex process. In the last decade, different theories were postulated to explain the unequal differentiating cleavage. Kochert proposed a modern version of Weismann's "germ-plasma"-theory, in which a cytoplasmatic, morphogenetic substance determines the differentiation [8]. Pall has correlated the size of the blastomeres with their potency to become gonidia [9]. This model does not solve, but only shifts the problem to the question: Why do some blastomeres become larger than others? And recently, Sumper put forward a sophisticated model, in which differentiation is established by cell-cell contacts by means of a sulfated membrane glycoprotein [10, 11].

Whereas most of the investigators have recognized the basic gonidial pattern, which consists of 16 or 8 gonidia in the *nagariensis* or *weismannia* strains of *Volvox carteri*, the mode of pattern variation was overlooked. Especially in *Volvox carteri* f. *nagariensis*, however, the basic pattern of 16 symmetrically arranged gonidia is found only in 15% of the individuals in optimal environment; the majority of the spheroids has fewer gonidia (down to 8). Under

\* Dedicated in friendship to Prof. Ernst Helmreich on occasion of his 60th birthday.

Reprint requests to Prof. Dr. L. Jaenicke.

0341-0382/82/1000-1023 \$01.30/0

ordinary conditions the formation of the basic gonidial pattern seems to be more the exception than the rule. A model, which seeks to explain the gonidial differentiation, should also explain the variability of the gonidial pattern; and, more important, the kind of variation should be correlatable to the mechanism of differentiation involved.

Let us explain the latter in detail. The analysis of spheroids containing less than 16 gonidia will show which gonidial stem cell fails to differentiate; thus pattern variation reflects differentiation defects. We expect three different modes of pattern variation:

(i) The defects are completely random. This would indicate that there is no interference between differentiation of the gonidial stem cells, *i.e.* an accidental drop-out in differentiation of one stem cell does not affect the others.

(ii) The defects are primarily random, but are propagated according to the ontogenetical relation of the gonidial stem cells. This would be the case if the differentiating cleavage is triggered by the presence or absence of a cytoplasmatic or membraneous substance. During cell cleavages this message has to be passed on to the blastomeres which give rise to the gonidia. A variation of the basic gonidial pattern must therefore be due to an interruption of the normal "flow" of information, and consequently, a missing link will block the information for subsequent blastomeres.

(iii) The defects are systematic. This must be due to an intrinsic mechanism which determines the kind of pattern variation. Gonidial numbers will, therefore, be represented by a predictable pattern, *i.e.* each number of gonidia is unambiguously correlated to a distinct gonidial pattern.

### Pattern Formation of the Gonidial Cells

*Volvox carteri* is a slightly egg-shaped hollow sphere swimming with the anterior end ahead, while rotating about its longitudinal axis. The gonidia are located in the posterior two thirds of the spheroid just beneath the somatic sheath, as shown in Fig. 1 (replacing one or several somatic units with their touching surface). The basic pattern formed ideally by 16 gonidia, is established with extraordinary invariability. Seen from the anterior pole, there are four quadrangles of four gonidia each on top of each other. In the side view one will notice that these

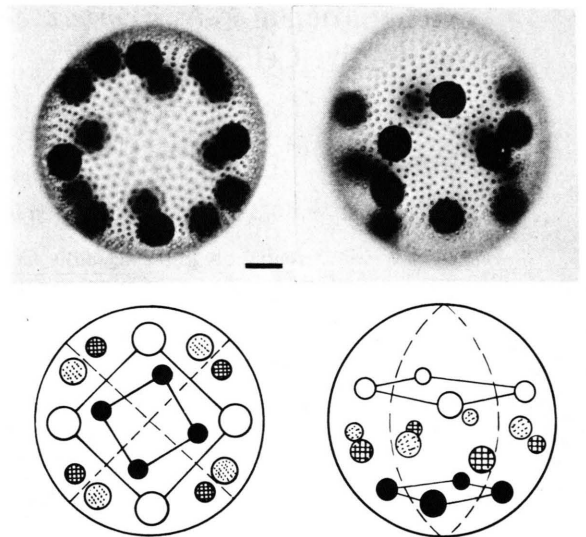


Fig. 1. Upper left: Spheroid of *Volvox carteri* f. *nagariensis* containing 16 gonidia. View from the anterior pole; the anteriormost layer of gonidia has been focused. Lower left: Schematic representation of the anterior view. Upper right: Side view of a 16-gonidia spheroid; some of the backside gonidia are completely out of focus. Lower right: Schematic representation of the side view. In the diagrams the gonidia are not to scale; their size indicates relative spatial position. Gonidia of one tier have equal marks. Closed circles: layer 1; hatched circles: layer 2; dotted circles: layer 3 and open circles: layer 4. Solid lines connecting the gonidia of the layer 1 and 4 exemplarily illustrate that each layer forms a quadrangle. Broken lines indicate the quarters derived from one blastomere of the 4-celled embryo. Bar: 50  $\mu$ m.

layers are planar and perpendicular to the pole axis. The corresponding four gonidia of each layer form diamonds since the lowest and top-most layers are nearly superimposed (actually the latter is twisted around the polar axis by about  $-10^\circ$ ) and the central layers are turned approximately  $35^\circ$  clockwise and counterclockwise, respectively, relative to the upper layer.

This basic pattern of gonidial cells can be deduced from the positioning of the gonidial stem cells during embryogenesis. Cell lineages and morphology of the embryo are well established by light and scanning electron microscopy [12–14]. Already at the 64-celled embryo (the stage just after unequal division) the gonidial pattern of the adult spheroid becomes evident; during further development it will merely be expanded by cleavage of the somatics and inverted by turning the embryo inside out during the process of inversion. From a developmental point of view, it

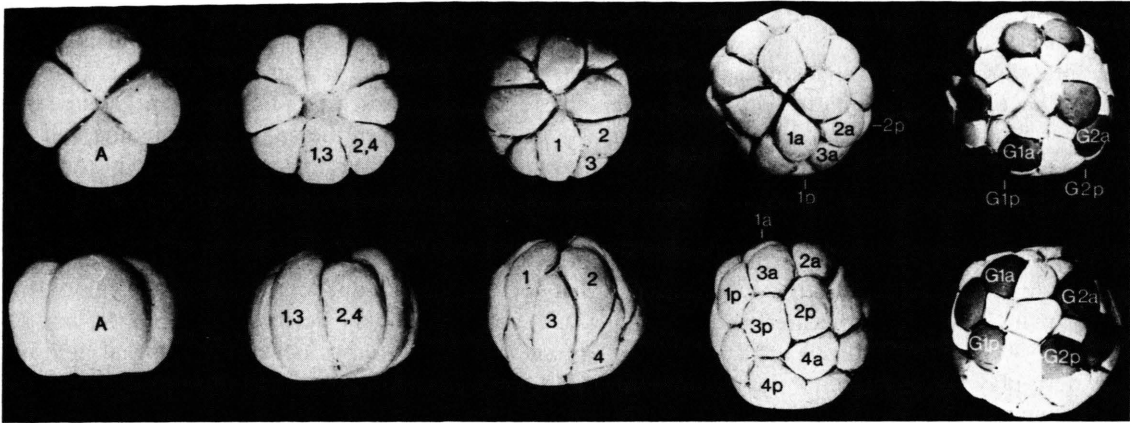


Fig. 2. Plasticine models of 4- to 64-celled embryos built on the basis of microscopical studies. The upper lane shows the anterior and the lower the side view. The nomenclature introduced by Green and Kirk is used to demonstrate the cell lineages. The gonidia (G) are labeled according to their stem cells. Note that the positioning of the gonidia in the 64-celled embryo is the inverted one of the adult spheroid.

is obvious that the four gonidia in each layer are not related ontogenetically, but rather each is related to the other three in that quarter of the spheroid which has arisen from the same primordial blastomere of the 4-celled embryo. A side view shows each quartet as an oblique, somewhat asymmetric lozenge the long diameter of which being at a defined negative angle to the pole axis (Fig. 1).

On further detailing, we ask which blastomeres give rise to the gonidia. In the following a short description of the embryogenesis will be presented on the basis of models of the 4- to the 64-celled embryos (Fig. 2).

The gonidium cleaves two times resulting in a 4-celled embryo, which already has a marked polarity, presented by an open anterior end becoming the phialopore and a posterior end at which the cells are in close contact. The third and fourth cleavages are oblique and therefore the 16-celled embryo consists of four extensively overlapping tiers. From the anterior two tiers (cells 1 and 2) the gonidia are formed, whereas the posterior ones develop exclusively of somatic cells. For this reason we may say that the fourth cleavage is *functionally* unequal. The fifth cleavage is the first equatorial one, which produces anterior and posterior sister cells. When the sixth, *differentiating*, cleavage occurs in the 32-celled embryo, only those 16 anterior blastomeres which have arisen from tiers 1 and 2 of the 16-celled stage undergo an unequal cleavage. As a result, the 64-

celled embryo consists of 16 large cells and 48 small cells. The gonidia are ultimately formed from these 16 large gonidial initials. During the subsequent 5 to 6 cleavages, the gonidial initials continue to divide unequally while the 48 small cells divide equally, eventually forming 2000 to 4000 somatic cells\*. As seen from Fig. 2 gonidial initials at the 64-celled stage show basically the same relative positioning as the gonidia of the adult spheroid. We must take into account, however, that on inversion the anterior end of the embryo becomes the posterior end of the spheroid, but the sides are not changed.

Now we are able to make the following correlation: The first gonidial layer (the posteriormost in the later daughter spheroid) results from unequal division of 1a cells, the second from 2a; the third from 1p and the fourth from 2p cells of the 32-celled embryo. It might be astonishing that the gonidial pattern is preserved during morphogenesis, but the gonidia, like all other cells, are crosslinked to their neighbouring cells by the cytoplasmic bridge system, which holds the embryo together and does not allow relocation of a single cell [13].

\* Actually, it becomes more and more difficult to follow the unequal cleavages of the gonidial initials morphologically during these stages, it seems, however, that the cleavages of the gonidial initials cease earlier than those of the somatic cells. Green and Kirk calculated from the total number of cells in the adult spheroid that the initials cleave only three times after the sixth cleavage [13].

### Variation of the Gonidial Pattern

In the preceding section we have assumed the spheroids to have always 16 gonidia. This is not true under either natural or laboratory conditions, but the number of gonidia per spheroid varies between 8 and 16. Fig. 3 illustrates the distribution of the number of gonidia in two different types of cultures. The number seems to depend on environmental conditions; it is lower in overcrowded, slowly growing cultures than in low density cultures in the logarithmic phase. The reason for this is not known; light intensity may play an important role. It is worth noting that in any case the distribution is not simply binominal, but in optimally growing cultures the numbers between 12 and 16 and under less optimal conditions the numbers between 12 and 8 are clearly preferred. Numbers below 8 and above 16 are found more rarely than expected from the statistics. Thus, the mechanism of variation is unable to increase the basic gonidial number of 16.

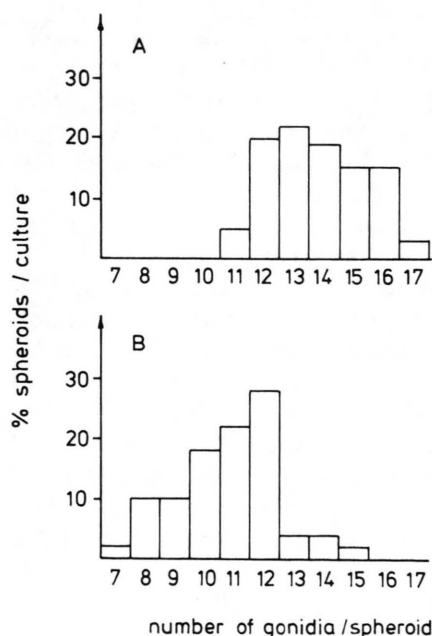


Fig. 3. A: Distribution of the number of gonidia per spheroid in a logarithmically growing culture at a density of 10 spheroids/ml. B: Distribution of the gonidial number in an overcrowded culture (> 1000 spheroids/ml). 400 individuals of each culture were counted. The conditions for all cultures used in this study are: synthetic *Volvox* medium as described in [15]; light:dark rhythm = 16:8 h; light intensity = 13 000 lux; temperature = 28 °C.

Let us now consider, how the gonidial pattern in spheroids containing less than 16 gonidia is established. The most important observation is that the basic pattern is preserved in any case, *i.e.* some sides or complete layers of the pattern are missing, but the position of the remaining gonidia is almost unaffected. Thus, it is reasonably easy to determine which gonidial stem cells failed to differentiate. Microscopical studies clearly show, that the drop-out of gonidia is really due to a failure in differentiation and not a secondary reversion. Up to 5% of the gonidial initials do not develop to normal gonidia, but form rudimentary cells, which can be seen as dark spots in the adult spheroid. In our study these rudimentary gonidia, which are randomly distributed, are always counted as normal ones, because they have been primarily differentiated and are not drop-outs. To classify the gonidia, each spheroid was examined microscopically in an anterior and in a side view; the combined results are shown in Fig. 4.

As outlined in the introduction, we may expect either a random or a systematical variation of the pattern. We found, indeed, a systematic mode of pattern reduction, in which each configuration can be predicted from the total number of gonidia alone. Starting from the 16-gonidiate spheroid, the first four drop-outs are always in the second layer, *i.e.* 2 *a* cells do not differentiate. In the four rhomboidal quartets the right sides are missing; thus the 12-gonidiate spheroid consists of four triangular trios of gonidia. Because the spheroids are radially symmetrical, we can, of course, not predict which 2 *a* gonidium will be missing in a 15-gonidiate spheroid. In case of the 14-gonidiate spheroid, two configurations, *para* and *ortho* are possible. Although only the *para* configuration is shown in Fig. 4, there is no preference of the *para* or *ortho* placement (27 and 23 individuals respectively), a fact which is not obvious, because in the 4-celled embryo only each *para* pair of blastomeres is identical. This asymmetry has, however, no effect on differentiation. If the gonidial number is lower than 12, the posteriormost layer of gonidia, 1 *a* gonidia, in addition is stepwise reduced. For the configuration of the 11- and 10-gonidiate spheroids, the same statements as for the 15- and 14- are valid. At the end of the pattern reduction an 8-gonidiate spheroid remains, constructed by two quadrangles, which are approx. at 45° to each other, derived from 1 *p* and 2 *p* cells.

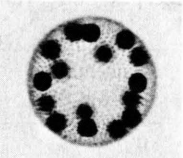
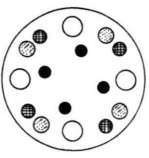
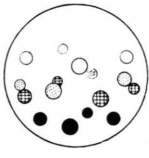
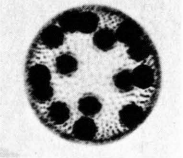
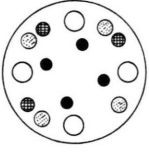
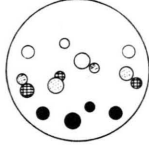
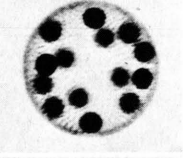
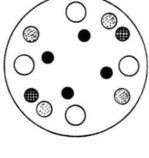
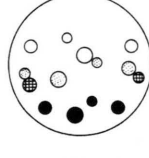
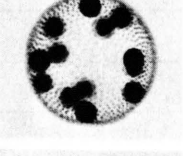
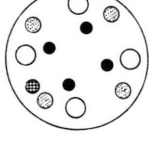
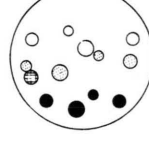
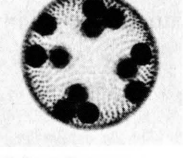
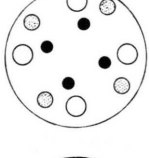

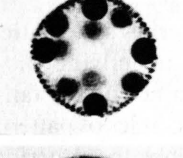
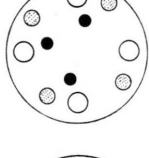
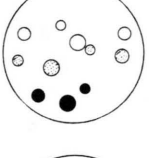

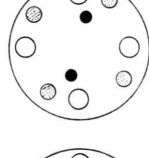
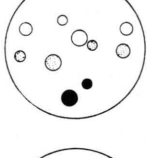

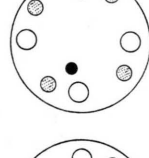
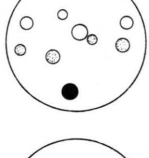

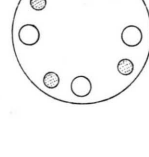
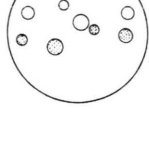
ANTERIOR VIEW	ANTERIOR VIEW	SIDE VIEW	NUMBER OF GONIDIA
			16
			15
			14
			13
			12
			11
			10
			9
			8

Fig. 4. Microscopical and schematic representation of the gonidial pattern of 16- to 8-gonidiate spheroids. The diagrams and micrographs are analogous to those in Fig. 1. In the diagrams the gonidial pattern is reduced simply by omitting the missing gonidia without changing the other gonidia positions. Therefore the positioning in spheroids containing less than 13 gonidia is not absolutely precise, because on total loss of one layer the others move somewhat more to the posterior end.



Table I. Statistical analysis of the gonidial pattern. Each pattern is classified by four digits indicating the number of gonidia per layer 1–4. Spheroids which show the predicted pattern are counted as “normal”, with the exception of “twins” which have five gonidia in one tier and are counted separately.

Type of pattern	Number of gonidia	Classification	Number of spheroids	Normal configuration	Abnormal configuration
regular	16	4444	41	41	0
	15	4344	37	36	1
	14	4244	51	50	1
	13	4144	70	68	2
	12	4044	81	81	0
	11	3044	63	60	3
	10	2044	48	48	0
	9	1044	33	32	1
	8	0044	37	36	1
			462 = 90.2%	452 = 88.3%	10 = 1.9%
twin	x	xxx5	5		
	x	xx5x	3		
	x	x5xx	2		
	x	5xxx	16		
			26 = 5.1%		
irregular	x	—	24 = 4.7%		
total			512 = 100%		

In Table I the various gonidial patterns are classified by four figures, each indicating the number of gonidia per tier 1–4. It is obvious, that each total number of gonidia is represented by a distinct gonidial configuration and figure composition. 88% of the analysed spheroids have a gonidial pattern as predicted. The other individuals can be divided into three types: One carries a duplication of a gonidium; these “twins”, as we call them, have one additional gonidium closely adjacent to a regular one. Both are smaller than the other gonidia, we therefore suppose that at the seventh or later division an equal, rather than an unequal cleavage of a gonidial initial occurs, resulting in two cells both capable of becoming gonidia. We found 5% twins, and – remarkably – all of the 17-gonidiate spheroids were twins. The tendency to form twins is more strongly marked in the 1a layer of gonidia. The other type has really a gonidial pattern which does not match the standard placement; these 2% “invalids” seem to be errors of the variation mechanism. At least 5% of the individuals have no symmetrical pattern at all, the percentage is inversely proportional to the quality of culture conditions. Perhaps the pattern deterioration is caused by a disturbance of the cleavages, for the arrangement of the somatic cells is also affected in

those spheroids which are often unable to swim normally.

### Mechanism of Pattern Variation

The variation of the gonidial pattern is systematic, thus it must be governed by an underlying biochemical mechanism and not by an accidental failure of differentiation. Two basic principles of pattern reduction become apparent: (i) Only the anterior derivatives of cells 1 and 2 are affected. (ii) The drop-outs of the 2a layer always precede those of the 1a layer.

No model of differentiation, which we are able to envisage, can reasonably explain, why e.g. 10 cells lying rather scattered in the 32-celled embryo should differentiate to form a 10-gonidiate spheroid; or why just 2a cells which form the second tier of the embryo will be omitted first in differentiation. In our opinion it is impossible to interpret the variation of the gonidial pattern by differentiation of a variable number of cells at the sixth cleavage, since direct observation convinced us that the mechanism of variation lies in a variability of the point of differentiating cleavage. Microscopical studies show

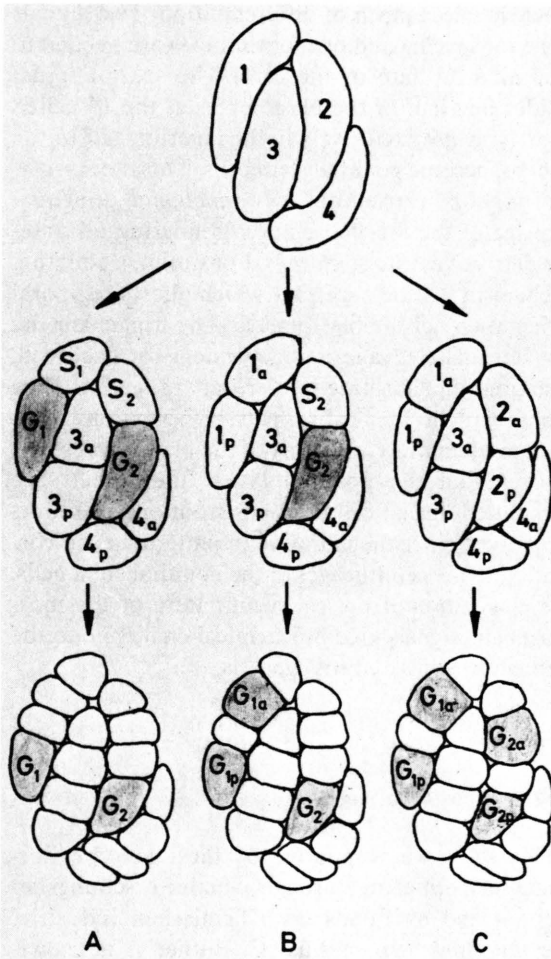


Fig. 5. Cleavage pathways from the 16- to the 64-celled embryo. Only one quarter is shown. The unequal cell divisions give rise to a gonidial (G) and a somatic initial (S). The nomenclature is as in Fig. 2. Note that the gonidial initials continue to cleave unequally.

that the 8-gonidiate spheroid is differentiated at the fifth rather than at the sixth cleavage. This fact was also recognized by Starr [4]. The 8 gonidia arise from an unequal cleavage of cells 1 and 2 of the 16-celled embryo. Thus, differentiation occurs at the 16-celled stage for the 8-gonidiate spheroid and at the 32-celled stage for the 16-gonidiate spheroid; and it is a reasonable assumption that the intermediate numbers of gonidia result from an asynchronous differentiation of the stem cells either at 16- or 32-celled stage. Fig. 5 demonstrates the different pathways of cleavages from the 16- to the 64-celled embryo. In addition to the two normal pathways (A

and C) a third pathway (B) is shown resulting in a 12-gonidiate spheroid. In this case differentiation of cells 2 occurs at the fifth cleavage, hence the 32-celled embryo contains already four gonidial initials (one per quarter). In the next cleavage also cells 1 which are meanwhile duplicated into 1a and 1p cells differentiate, and the 64-celled embryo contains 12 gonidial initials. If we combine this new pathway with one of the basic pathways assuming that the four cells of one tier will be differentiated independently of each other, we will be able to explain the occurrence of the 8- to 12- or the 12- to 16-gonidiate spheroids. We can, therefore, deduce each of the gonidial numbers from a simple shift of the differentiating cleavage. The gonidial stem cells differentiate either at the 16- or at the 32-celled stage. There is no interference, with the exception of priority of cells 2. In order to understand why the gonidial pattern is not affected by the non-synchronous differentiation, we must realize the following:

The unequal division takes place in such a way that the large initial is always located more posterior than the small one. Thus, the gonidial initial is in the same position as the posterior derivative at an equal division. It is obvious that our previous correlation between the stem cells and the gonidia must be more precisely stated according to the new pathways; e.g. the gonidia of a 12-gonidiate spheroid arise not from 1a, 1p and 2p cells, but from 1a, 1p and 2 cells.

### Concluding Remarks

The mechanism of pattern variation described is extraordinarily satisfying, since it does not only explain the different patterns formed, but also the unusual distribution of the gonidial number. Only numbers between 8 and 16 can be generated by the mechanism and approximately all of the individuals contain those numbers of gonidia. The fact that the numbers fall into two classes, 8 to 12 and 12 to 16, makes now sense, for they are formed by two different combinations of pathways (A and B or C and B). It might seem dissatisfying to deviate from the synchrony of differentiation, but variation of the gonidial pattern, unavoidably involves change of one of the basic parameters, either point of differentiation or potency of cells. In the latter case we must explain, why do some of the stem cells fail to differentiate, a fact which would be at least as trouble-

some as the former and no observation indicates preferentially such a restriction in potency of the stem cells to become gonidia. In our opinion it is reasonable from a developmental point of view that despite of the total number the gonidia always derive from *all* four cells 1 and 2 of the 16-celled embryo. These cells are obviously determined by any mechanism to become gonidia and only the point of their differentiation is not strictly fixed. The temporal freedom of the first unequal cleavage is not unparalleled, since the cessation of all cleavages in embryogenesis varies as well.

Another point is the principal difficulty to define what is meant by differentiation at a cellular level. In this communication we use the terms "differentiating cleavage" and "unequal cleavage" as synonyms; by so doing we follow the common use of previous workers [3, 10, 14]. Nevertheless we keep in mind that "unequality" in cleavage is a morphological term, whereas "differentiation" refers to a developmental program. The problem in *Volvox carteri* is that the gonidial initials undergo more than one unequal division; thus we are unable to spot morphologically the exact stage of differentiation. In fact, differentiation is not a sudden step in embryogenesis, but a process starting (at least) with the *first* unequal cleavage and ending with the formation of the gonidium. For as long as we have no other marker we should be permitted to see in the first unequal cleavage the first visible manifestation of differentiation.

Our observations on and explanation of the gonidial pattern variation enables us to make some points

about the mechanism of differentiation. Two signals — one for spacing and one for timing — are needed to determine the fate of the cells. The spatial signal decides that half of the blastomeres at the 16-celled stage, lying *not* exclusively in the anterior half of the embryo, become gonidial stem cells. This determination might be carried out by a *functionally* unequal cleavage of the 8-celled embryo rendering all anterior derivatives into stem cells. For timing a counting mechanism will be necessary which allows temporal "orientation" of the blastomeres. The trigger for the first unequal cleavage is independent for each cell; assuming the absolute preference of cells 2, one might explain the entire pattern variation by a random premature triggering of the blastomeres. We can rule out the possibility that the signals are transmitted during cell divisions from one cell to its derivatives, since the variation in pattern has no correlation to the cell lineages of the gonidial stem cells. The elucidation of the chemical nature of the morphogenetic signals give biochemical challenge to the problem in *Volvox* embryogenesis.

#### Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 74 and by Fonds der Chemischen Industrie. The unfailing help of Miss C. Bittner is acknowledged. We thank Prof. R. C. Starr, Austin, for critically reading and discussing this manuscript.

- [1] A. Weismann, The continuity of the germ-plasm as the foundation of heredity. In: Essays upon heredity (E. B. Poulton, S. Schonland, and A. E. Shipley, eds.). Clarendon Press, Oxford 1889.
- [2] L. Jaenicke, TIBS 7 (2): 61–64 (1982).
- [3] R. C. Starr, Proc. Natl. Acad. Sci. USA 70, 1335–1338 (1968).
- [4] R. C. Starr, Arch. Protistenkd. 111, 204–222 (1969).
- [5] G. I. Viamontes and D. L. Kirk, J. Cell Biol. 75, 719–730 (1977).
- [6] G. I. Viamontes, L. J. Fochtmann, and D. L. Kirk, Cell 17, 537–550 (1979).
- [7] L. Jaenicke and S. Waffenschmidt, Ber. Deutsch. Bot. Ges. 94, 375–386 (1981).
- [8] G. Kochert and I. Yates, Dev. Biol. 23, 128–135 (1970).
- [9] M. L. Pall, in Developmental Biology: Pattern formation, Gene regulation. ICN-UCLA Symposia on Molecular and Cellular Biology. (D. Macmahon and C. Fred Fox, eds.). W. A. Benjamin, Inc. 2, 148–156 (1975).
- [10] M. Sumper, FEBS-Letters 107, 241–246 (1979).
- [11] S. Wenzl and M. Sumper, Proc. Natl. Acad. Sci. USA 78, 3716–3720 (1981).
- [12] R. C. Starr, Dev. Biol. Suppl. 4, 59–100 (1970).
- [13] K. L. Green and D. L. Kirk, J. Cell Biol. 91, 743–755 (1981).
- [14] K. J. Green and D. L. Kirk, J. Cell Biol., submitted for publication.
- [15] R. C. Starr and L. Jaenicke, Proc. Natl. Acad. Sci. USA 71, 1050–1054 (1972).