THE EXTRACELLULAR MATRIX OF VOLVOX:
A COMPARATIVE STUDY AND PROPOSED SYSTEM OF NOMENCLATURE

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SUMMARY
The structure of the extracellular matrix (ECM) of representatives of all four sections of the
genus Volvox was examined by a combination of light- and electron-microscopic methods. On the
basis of these observations, plus published descriptions of aspects of ECM organization in other
members of the order Volvocales, a system of nomenclature is proposed, to facilitate discussion of
comparative morphology and phylogeny of the ECM in the order. In this system the ECM is
divided into four main zones: the flagellar zone (FZ), which consists of attachments to and special-
izations of the ECM around the flagella; the boundary zone (BZ), which consists of portions of the
ECM that (except in periflagellar regions) are continuous over the surface of the organism and are
not structurally continuous with deeper layers; the cellular zone (CZ), which consists of
specializations, other than those of the FZ, around individual cells; and the deep zone (DZ), which
consists of components that fill the central region of the organism, internal to CZ. An empirically
based set of hierarchical subdivisions of these zones is then proposed that permits specific identi-
fication of most morphologically distinct ECM components. The fact that not all zones and
subzones are present in all members of the order means that this system permits identification of
those ECM structures that have been gained or lost during Volvocalean evolution.

Species-specific differences in the structure of virtually all aspects of the ECM were seen among
the Volvox species examined in this study. However, the fact that such differences cannot always be
used as diagnostic characters for the four divisions of the genus was demonstrated by the obser-
vation that in certain ECM features two members of the same division (V. carteri f. nagarensis and
V. carteri f. weismannia) differ markedly in structure from one another, with one member of the
pair resembling a member of another division. Thus many details of ECM organization appear to
be under separate control, and capable of independent evolution.

INTRODUCTION
It is now widely recognized that many important developmental and physiological
responses of metazoan cells are mediated by the extracellular matrix, or ECM, with
which those cells are in contact (Hay, 1981; Piez & Reddi, 1984; Trelstad, 1984).
The extent to which higher plant cell walls constitute an ECM with structural
parallels to the metazoan counterpart has been clearly articulated by Lamport
(1974, 1977, 1980): both are based upon fibrous hydroxyproline-rich glycoproteins

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(the collagens of animals and the extensins of plants), associated with complex mixtures of carbohydrates and proteoglycans. The plant cell wall has long been recognized as being fundamentally important in regulating morphogenesis in a biophysical manner (Green, 1980), but its possible centrality as a chemical mediator of the effects of plant hormones and growth regulators has only been recognized more recently (McNeil, Darvill, Fry & Albersheim, 1984). Thus the assessment Hay (1981) made with respect to metazoa should probably be generalized to all higher eukaryotes: 'For a full understanding of cell biology, then, we need to know the compositions and sources of the extracellular matrices, the manner in which they are produced and secreted by cells, and the mechanisms by which these extracellular molecules interact with cells to affect their growth and metabolism'.

Several considerations recommend the green flagellates of the order Volvocales as a particularly favourable model system for analysing both the ontogeny and the phylogeny of a complex ECM. The Volvocales range in complexity from unicellular Chlamydomonas through colonial genera of increasing size (such as Gonium, Pandorina and Eudorina) to multicellular organisms, with differentiated cells and division of labour, in the genus Volvox. Each individual in the order contains 2n Chlamydomonas-like cells (with the maximum value of n being a species character) held together in a predictable pattern by a hydroxyproline-rich ECM. Elaboration of the ECM is a major basis for the increases in size seen in both the phylogeny (Bell, 1985) and the ontogeny of the more complex forms (Starr, 1970a). Furthermore, recent studies demonstrate that, as in higher plants and animals, the Volvocalean ECM is actively modified in developmentally specific ways that reflect and, or, determine the developmental behaviour of the cells embedded in it (Gilles, Gilles & Jaenicke, 1983, 1984; Wenzl, Thym & Sumper, 1984).

The most conserved morphological feature of the Volvocalean ECM is a 'tripartite' layer that in every species yet examined surrounds the entire individual (except where penetrated by flagella), whether the individual be unicellular or multicellular. Roberts and his associates have shown that in C. reinhardii this region is composed of a number of hydroxyproline-rich glycoproteins in a distinctive crystalline lattice that may be disassembled by chaotropic agents and recrystallized in vitro (Catt, Hills & Roberts, 1976, 1978; Hills, Phillips, Gay & Roberts, 1975; Roberts, 1974, 1979; Roberts, Gay & Hills, 1980). Immunological and genetic dissection of this structure is well under way (Davies & Roberts, 1976; Smith, Roberts, Hutchings & Galfre, 1984). All multicellular Volvocales yet examined possess crystalline lattices that are extremely similar to that of C. reinhardii, but different from those of many other green algae (Roberts, 1974; Roberts, Shaw & Hills, 1981); furthermore, the crystalline layer of the Volvox carteri ECM has recently been recrystallized in parallel with that of C. reinhardii and shown to consist of a similar set of glycoproteins, all of which are slightly different in size in the two species (Goodenough & Heuser, personal communication).

The inner portion of the Chlamydomonas cell wall is an insoluble, relatively undistinguished-looking fibrous network (Roberts, 1974; Goodenough & Heuser, 1985). But it is apparently within the evolutionary derivatives of this
Volvox extracellular matrix

undistinguished-looking layer that diversification has occurred to convert a simple cell wall into an ECM that made possible the larger and more complex bodies of the higher Volvocales.

In the higher Volvocales the ECM internal to the crystalline layer is organized in highly regular, species-specific patterns into a series of fibrous layers that underlie the crystalline layer, ensheath individual cells, surround cells at a distance to form contiguous cellular compartments, and subdivide the deeper regions into distinct zones. Spaces defined by these fibrous layers are filled with relatively amorphous, mucilaginous components. Selected aspects of ECM organizational patterns have been provided at the light- and, or, electron-microscopic level for a number of genera, species, strains and developmental stages (Birchem, 1977a,b; Birchem & Kochert, 1979; Bisalputra & Stein, 1966; Burr & McCracken, 1973; Darden, 1966; Dauwalder, Whaley & Starr, 1980; Deason, Darden & Ely, 1969; Domozycz, Stewart & Mattox, 1981; Fulton, 1978a,b; Gottlieb & Goldstein, 1977; Kochert, 1968; Kochert & Olson, 1970; McCracken & Barcellona, 1976, 1981; McCracken & Starr, 1970; Pickett-Heaps, 1970, 1975; Sessoms, 1974; Smith, 1944, 1950; Starr, 1969, 1970a,b; Tautvydas, 1978; Vande Berg & Starr, 1971), but a comparative study at the EM level has not previously been reported.

Chemical and genetic analysis of the ECM of the higher Volvocales has been started. A hydroxyproline-rich glycoprotein and a mannose-rich polysaccharide have been isolated from Eudorina (Tautvydas, 1978) and a sulphated polysaccharide has been isolated from Platydorina (Crayton, 1980). But the greatest progress has been made with analysis of the ECM of V. carteri. As mentioned above, analysis of the crystalline layer is in progress. In addition, two interesting hydroxyproline-rich glycoproteins from the deeper regions have been purified and analysed; one is a major component of the ECM that appears to be a polyhydroxyproline (Mitchell, 1980); the other is a glycoprotein that is sulphated in a manner that reflects reproductive cell development (Wenzl et al. 1984). Other secondary ECM modifications related to sexual development in this species have also been described (Gilles et al. 1983, 1984), and the enzyme believed to be involved in dissolution of the parental ECM during release of juvenile spheroids has been isolated (Jaenicke & Waffenschmidt, 1979). At least six V. carteri loci have been defined at which mutation leads to ECM defects (Huskey, Griffin, Cecil & Callahan, 1979; Sessoms, 1974; Sessoms & Huskey, 1973), and DNA sequences with the potential to code for hydroxyproline-rich proteins have been cloned and sequenced (Kirk & Harper, 1985). Furthermore, monoclonal antibodies specific for a number of discrete ECM components have been prepared (Hoffman, 1984; Hoffman & Kirk, unpublished data), permitting correlations to be made between defined macromolecules and morphological regions of the ECM.

In short, most of the methods required for analysing the molecular—genetic foundations of the ECM in one of the simplest Volvocales, Chlamydomonas, and one of the most complex, V. carteri, have been or are being developed. Together these developments indicate that the Volvocales provide a unique and exploitable opportunity for exploring the genetic basis for evolution of a complex ECM.
One barrier to progress, however, has been the lack of a consistent nomenclature to describe the Volvocalean ECM. Over the years a plethora of inconsistent terms has been used by various investigators. Certain ECM structures have been given entirely different names by different observers; on the other hand certain terms have been widely used, but in entirely different ways by different investigators. As an example of the latter, some investigators have used the terms 'sheath' and 'matrix' interchangeably, while others have made a clear distinction between the two – but the distinctions drawn in different studies bear little resemblance to one another.

A major goal of the present study, therefore, was to develop a system of nomenclature, based upon comparative observations, that might be widely applicable for description of the organizational features of the Volvocalean ECM, and that might facilitate future communication among investigators exploring problems of mutual interest in different species or with different methods. To that end, we have performed a comparative light- and electron-microscopic study of the ECM architecture in the most complex members of the order. More specifically, we have examined representatives of the four major divisions of the genus *Volvox* that Smith (1944) recognized, based partly on the differences in ECM organization that are apparent by light microscopy. These were (with division names given in parentheses): *V. globator* (Euvolvox), *V. aureus* (Janetosphaera), *V. dissipatrix* (Copelandosphaera), and *V. carteri* forma *nagariensis* and forma *weismannia* (Merrillosphaera). In developing the proposed descriptive terminology we have also taken into account published reports (listed above) dealing with aspects of ECM organization in other species and genera, our goal being to make the terms as generally applicable as is presently possible. It should be recognized, however, that most of the studies included in this survey involved examinations of fixed, stained and sectioned material, and that any system of nomenclature based on such studies may ultimately require modification to accommodate results obtained with alternative modes of sample preparation and examination (such as the quick-freeze, deep-etch methods of Goodenough & Heuser, 1985), which tend to highlight different features of ECM organization.

**MATERIALS AND METHODS**

**Cultures**

*V. carteri* f. *weismannia*, strain Ka-1, was obtained from G. Kochert, and some studies of it were performed in the Kochert laboratory, using cultivation and preparation methods previously described (Birchem & Kochert, 1979); the remainder were performed in this laboratory in parallel with those of the other species. *V. globator* (UTEX LB 955), *V. aureus* (UTEX LB 1899), *V. dissipatrix* (UTEX LB 1871) and *V. carteri* f. *nagariensis*, strain HK 10 (UTEX LB 1885), were obtained from the Culture Collection of Algae at the University of Texas. Conditions for maintenance of *V. carteri* have been described (Kirk & Kirk, 1983); the other species were maintained similarly, except at 24°C rather than 32°C.

**Preparation for microscopy**

In most cases specimens were prepared by a variant of the DAB (3,3'-diaminobenzidine·4HCl) treatment which Burr & McCracken (1973) have shown brings out certain details of *Volvox* ECM
Volvox extracellular matrix

not seen by other preparative methods: organisms at selected developmental stages were fixed by the semi-simultaneous glutaraldehyde/osmium method described by Viamontes, Fochtmann & Kirk (1979); fixation was terminated after 15 min by removing most of the fixative and flooding the specimen with 1% (v/v) DAB in 5% boric acid for 30–40 min. Specimens were then treated with 2% OsO4 for 15 min, washed, dehydrated with ethanol and embedded in Spurr’s low-viscosity resin. Thick sections were examined with a Zeiss photomicroscope I, and thin sections were examined on Formvar-coated, slotted grids in a Hitachi HU-11C electron microscope.

Alternatively, the procedure of Luft (1966) for Ruthenium Red staining of acid mucopolysaccharides was followed: specimens were fixed for 30 min at room temperature and 6 h at 0°C in a 1:1 (v/v) mixture of 0.5% Ruthenium Red and 4% glutaraldehyde at pH 7.5, post-fixed for 11 h at 4°C with 1% OsO4, dehydrated with ethanol and acetone, embedded in Epon/Araldite and sectioned.

RESULTS AND DISCUSSION

General features of the Volvox ECM

V. globator, a member of the section Euvolvox, will be used as the type species to illustrate the general organizational features of Volvox ECM. As is true throughout the genus, individuals of this species possess several thousand cells embedded at the periphery of an otherwise transparent spheroid (Fig. 1). Most of the cells are of a rather uniform, small size and are post-mitotic somatic cells; the remaining few, somewhat larger cells are ‘gonidia’, or asexual reproductive cells, which have the capacity to divide and give rise to new juvenile individuals similar in organization to the adult. In living specimens, viewed at moderate power with the light microscope, the cells are seen to be connected by cytoplasmic strands to neighbours on all sides (Fig. 2). (Cytoplasmic connections formed during embryogenesis are subsequently lost in species in the section Merrillosphaera, but retained in adults in other sections of the genus.) Cross-sections of spheroids that have been fixed, stained with DAB and thick-sectioned for light microscopy reveal clearly the general outlines of the ECM that binds the cells together (Figs 3, 4): a layer of electron-dense material surrounds the spheroid, penetrated by the paired flagella of each cell; beneath this layer, each cell is enclosed in a complete, individual cellular compartment, with side walls shared between neighbouring compartments; beneath the cellular layer lies a meshwork of coarse, fibrous material that surrounds the relatively amorphous material in the centre of the spheroid. Although gonidia are initially confined to the same zone as the somatic cells (Fig. 3), the embryos produced from these gonidia enlarge between successive divisions and protrude progressively further into the central zone (Fig. 4).

Additional details of these ECM features are clearly revealed by electron-microscopic examination of thin-sectioned material (Fig. 5). At this level of resolution the boundary of the spheroid is seen to be composed of several morphologically distinct layers: the outermost portion of the boundary zone is a brushwork of long, electron-dense fibres, similar to those originally observed by Burr & McCracken (1973) in the closely related species, V. rousseletii. This brushwork is attached to and projects from the ‘tripartite’ layer, which, as discussed in the Introduction, is the most conserved feature of the Volvocalean ECM. Internal to the latter is a broad feltwork of densely packed fine filaments, and internal to that is a thinner zone of
Fig. 5. Electron micrograph of DAB-stained, thin-sectioned *V. globator* spheroid, illustrating organization of ECM in the vicinity of a somatic cell. Note differing textures of filaments in different regions of the ECM. ×10 000.

Figs 1–4. Light micrographs illustrating organizational features of *V. globator*.
Fig. 1. Bright-field micrograph of living spheroid. The larger cells (g) are gonidia (asexual reproductive cells); the remaining cells are terminally differentiated somatic cells. ×180.
Fig. 2. Nomarski differential interference contrast micrograph of somatic cell monolayer of a living spheroid, showing cytoplasmic bridges (arrows) that connect neighbouring cells. ×700.
Fig. 3. Bright-field micrograph of a thick section through a DAB-stained spheroid. Larger arrow indicates a gonidium; smaller arrows indicate flagella of selected somatic cells. Note coarse fibrous layer underlying cells. ×380.
Fig. 4. Higher-power view of a specimen similar to that above, but with two embryos (derived from gonidia) in different developmental stages. Note penetration of fibrous layer by enlarging embryos. ×980.
slightly coarser filaments that can be seen to be continuous with the filaments that form the walls and floors of the cellular compartments. The plasmalemma of the cell is coated with a thin, coherent meshwork of similar filaments; this 'cellular envelope' or 'vesicle' is thicker and more obvious in other species (as will be seen in later figures) but is clearly present in this species as well. The space between the compartment boundary and the cellular envelope is filled with a loose array of filaments, a few of which appear to be continuous with either the compartment boundary or the cellular envelope. In the region where the flagellum extends to the exterior of the spheroid, a number of specializations of the ECM are seen that will be discussed in more detail later.

A proposed system of nomenclature for the Volvox ECM

It is clear from the foregoing brief discussion that the ECM architecture of a single species of *Volvox* is quite complex, consisting of a large number of anatomically distinct structures in a defined spatial relationship. Discussion of similarities and differences in such structures among species should be facilitated by a set of terms that could be used to focus the discussion on one defined anatomical feature at a time, in some kind of systematic manner. Ideally, such a system of terminology should have the following qualities. It should be as simple as possible, given the complexity of the details to be encompassed. It should be devoid of terms (such as 'sheath' *versus* 'matrix'), which have been widely but imprecisely used in the past and have been given entirely different connotations by different investigators. Because its purpose is to describe morphological features in a framework that can subsequently be used in discussions of comparative biochemistry, it should be based on anatomical terms that are neutral with respect to possible chemical similarities or differences among structural elements. It should be general enough to be readily applicable, with simple modifications, to descriptions of the ECM organization of all members of the order Volvocales. At the same time, it should be specific enough to permit precise identification of all morphologically distinct ECM components in each species. And it should be flexible enough to accommodate readily new information concerning ultrastructural details without major revision.

Our candidate for such a system is outlined in Table 1 and illustrated diagrammatically in Fig. 6. The basis for this scheme is simply stated. We propose that for purposes of comparative anatomy the ECM be subdivided into four major zones: the flagellar zone (FZ), the boundary zone (BZ), the cellular zone (CZ) and the deep zone (DZ); each of these major zones may then be subdivided in a hierarchical fashion to define observable substructures.

The proposed subdivisions have an entirely empirical basis: they are those that our present incomplete understanding of Volvocalean ECM organization suggests would be most useful. But as observations and understanding change, refinements of the hierarchy could be made readily, with no disruption to the scheme as a whole. Justifications for the particular subdivisions we have proposed will be included in the discussion that follows in the next section, in which this framework is used to discuss the comparative ECM anatomy of the species of *Volvox* that we have examined.
In the course of that discussion, it will become obvious that not all members of the order possess structures in each zone and subzone that we have proposed. We consider this a positive attribute of the scheme, because it aids in identifying ECM features that appear to have been gained or lost at various stages of Volvocalean diversification.

Because the proposed terminology is morphologically based, no judgements about chemical similarities or differences among the various components are implied; nor should any be inferred until or unless they have been demonstrated experimentally. However, once available, such chemical information could be used to refine the terminology so that it would recognize demonstrated homologies.

**Comparative anatomy of Volvox ECM**

In this section we will review, zone-by-zone, the details of ECM architecture in the representative species of Volvox that we have examined, to demonstrate the utility of

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<th>Table 1. Definitions of zones and selected sub-zones of the Volvocalean ECM</th>
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<tr>
<td><strong>FZ</strong> (flagellar zone): ECM specializations seen only on or in the immediate vicinity of the flagellum</td>
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<td>FZ1: coatings and appendages of the flagellar membrane (e.g. mastigonemes, sexual agglutinins, etc.; like all other zones and subzones, may be further subdivided as observations warrant)</td>
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<td>FZ2: component(s) attached to both the flagellum and FZ3, but separable from both (i.e. the flagellar collar)</td>
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<td>FZ3: modifications of the boundary and, or, cellular zones in the region where traversed by the flagellum</td>
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<td>FZ3a: specializations of BZ and, or, CZ that project outward, beyond the level of the rest of BZ2, in the flagellar region (i.e. the flagellar hillock)</td>
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<tr>
<td>FZ3b: specializations of BZ and, or, CZ around the flagellum, between the level of BZ2 and the cell body (i.e. the flagellar tunnel)</td>
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<td><strong>BZ</strong> (boundary zone): those components of the ECM that, except in periflagellar regions, appear to be continuous over the surface of the organism, but are not structurally continuous with deeper layers</td>
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<td>BZ1: the component(s) external to BZ2</td>
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<td>BZ2: the tripartite layer, corresponding to layers W2–W6 in the terminology of Roberts (1974), and containing the crystalline layer. This is used as the point of reference for the boundary zone because of its highly conserved nature</td>
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<tr>
<td>BZ3: the BZ component(s) internal to BZ2</td>
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<tr>
<td><strong>CZ</strong> (cellular zone): components, other than those of the FZ, lying internal to the boundary zone and exhibiting specializations around individual cells (in unicellular Volvocales the boundary zone and cellular zone are synonymous)</td>
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<tr>
<td>CZ1: the coherent meshwork of ECM filaments attached to the plasmalemma of each cell body (i.e. the cellular envelopes or vesicles)</td>
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<tr>
<td>CZ2: the relatively amorphous component(s) filling all portions of the CZ not occupied by more highly structured components</td>
</tr>
<tr>
<td>CZ3: the fibrous component that lies under the BZ, but is reflected inward around individual cells to create the compartment boundaries</td>
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<tr>
<td><strong>DZ</strong> (deep zone): all ECM components internal to the cellular zone</td>
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<tr>
<td>DZ1: a fibrous layer enclosing DZ2</td>
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<tr>
<td>DZ2: a relatively amorphous component filling the deepest regions of the spheroid (not present in all colonial species, but by far the largest region of the spheroid in most Volvox species)</td>
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Fig. 6. Stylized drawing of a portion of a *V. globator* spheroid illustrating ECM zones and sub-zones defined in Table 1; abbreviations as in that table. (Gonidal flagella are not shown, although they are present in this species before gonidia are mature.)
the proposed system of nomenclature in organizing comparative studies of this complex system.

The flagellar zone. All species of Volvocales examined (by us or others) possess numerous specializations of the ECM in the periflagellar region (Figs 7–14). Although all species also possess flagellar appendages, the inclusion of flagellar appendages as part of the ECM (FZ1) may appear to be something of a departure. Justification for this is provided, however, by recent studies by Goodenough, Adair, and their associates. They have added an entirely new dimension to the study of the Volvocalean ECM with the demonstration that the sexual agglutinins of *Chlamydomonas* (which are flagellar appendages by which gametes of opposite mating type recognize each other and couple before fusion) resemble components of the crystalline layer of the ECM both in their hydroxyproline-rich-glycoprotein nature (Cooper *et al.* 1983) and their three-dimensional structure (Goodenough, Adair, Collin-Osdoby & Heuser, 1985; Goodenough & Heuser, 1985) and are almost certainly members of the same protein family. In view of the generality of flagellar interaction as a sexual recognition mechanism in the Volvocales (Coggin, Hutt & Kochert, 1979; Coleman, 1979), such observations are likely to be extended eventually to other species. (Indeed, Goodenough (1985) has used these observations as the basis for a thought-provoking discussion of the role of such hydroxyproline-rich glycoproteins, and their genes, in the evolution of sex.)

When Ruthenium Red-stained specimens are sectioned parallel to the flagellar axis, a relatively uniform layer of intensely stained material is seen to coat the flagellum from its base to a point well beyond the level of the tripartite layer of the boundary zone, BZ2 (Fig. 13, large arrow). Following treatment with DAB this region is less intensely stained, but nonetheless an electron-dense structure of equivalent length is frequently seen (Figs 7, 8). In addition, numerous kinds of filamentous appendages of the flagellum are regularly observed (e.g. see Fig. 10). Although we have observed differences among the flagellar appendages of different *Volvox* species, the preparative and observational methods that we used did not include those that have been used with *Chlamydomonas* to generate high-

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Figs 7–10. EMs of equivalent sections from four DAB-stained specimens to illustrate differences in FZ, BZ and CZ structures in species representing the four sections of the genus *Volvox.* In these and all subsequent figures, all labels not explained in captions are defined in Table 1.

Fig. 7. *V.* globator. Note particularly the organization of FZ3a and BZ1 regions. In this figure and the next one the arrows without associated letters indicate an electron-dense coating of the flagellar membrane, which extends from the flagellar base to a region well beyond the level of BZ2. ×36000.

Fig. 8. *V.* dissipatrix. Note absence of FZ3a and continuation of BZ2 into the flagellar channel. Also note the much greater length of FZ3b, and the absence of a clear boundary between BZ3 and CZ3 in this species. ×43 000.

Fig. 9. *V.* aureus. Note that BZ2 does not appear to continue over the surface of FZ3a, and that CZ3 has an entirely different structure here from that in the other species. Note also the great length of FZ3b here. ×45 000.

Fig. 10. *V.* carteri f. nagariensis. Note abundant FZ1, scanty BZ1 and unique structure of FZ3a. ×36 000.
Figs 7–8. For legends see p. 217
Figs 9–10. For legends see p. 217
resolution images of such appendages (Goodenough et al. 1985). Therefore, detailed comparative analysis of _Volvox_ FZ1 awaits future studies of appropriate design.

The justification for creating a separate subzone, FZ2, for the flagellar collar is based on published observations of its behaviour in _Chlamydomonas_ (Roberts, Phillips & Hills, 1975; Snell, 1983). It is a thin cylinder, composed of a number of distinctive subunits in a crystalline array, that lies between and is attached to (but separable from) both the flagellar membrane and the remainder of the ECM. During resorption of the flagella before vegetative cell division, the collar remains attached to the 'flagellar tunnel' (FZ3b) and co-isolates with 'the mother wall' (Johnson & Porter, 1968; Roberts et al. 1975). In contrast, when flagella are removed from the cells by brief pH shock, the collar detaches from the flagellar tunnel and co-isolates with the flagella (Snell, 1983). During the mating reaction between gametes of opposite mating type, on the other hand, FZ2 detaches both from the flagellum and the tunnel, slides up the flagellum and off the tip, following which it may be isolated in pure form from the medium (Snell, 1983). Ruthenium Red staining of _Volvox_ brings out some of the substructure of FZ2 strongly (Fig. 11) and reveals it to have 56 subunits in transverse section, which is also the modal number in _Chlamydomonas_ (Ringo, 1967; Roberts et al. 1975). In these specimens, FZ2 appears firmly attached to FZ3b (the flagellar tunnel), but in addition, fine filaments can be seen connecting each FZ2 subunit to the flagellar membrane in the transition region between the axoneme and basal body, i.e. at the level of the star body (Fig. 12). Detailed comparative analysis of FZ2 also awaits future studies of appropriate design.

Among the most striking species-specific specializations of the ECM are those seen in FZ3a, the 'flagellar hillock'. In the representative of the Euvolvox section of the genus, _V. globator_, FZ3a has the form of a steep, hollow (volcano-like) conical structure, both surfaces of which are lined by continuations of BZ2 (the tripartite portion of the boundary zone); the core of FZ3a is filled with fairly homogeneous, electron-dense material, different in texture from anything seen elsewhere in the boundary zone (Fig. 7). Published micrographs of another species in the section _Euvolvox_, _V. rousselettii_, show it to have a quite similar type of FZ3a structure,

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Figs 11–15. EMs of selected portions of _V. carteri_ f. _weismannia_. Except where otherwise indicated, specimens were stained with Ruthenium Red.

Fig. 11. Transverse section through the paired flagella of a somatic cell, near the base of the axoneme. Note regularly spaced subunits of FZ2 and its apparent continuity with FZ3b. ×93,000.

Fig. 12. Similar section, but at the level of the star body (in the transitional region between the axoneme and basal body). Note fine filaments connecting each subunit of FZ2 to the flagellar membrane (arrows). ×127,000.

Fig. 13. Longitudinal section through the flagellum to illustrate the intense staining of the proximal flagellar membrane (larger arrow) (cf. Figs 7, 8). Also note deposit of stain on outer edge of BZ1 (smaller arrows). ×30,000.

Fig. 14. DAB-stained specimen, to permit comparison with the related forma shown in Fig. 10. In contrast to the latter, note (as in Fig. 12) the absence of FZ3a and the much more extensive BZ1, with a coherent outer edge (arrows). ×35,000.

Fig. 15. Section through the boundary zone to illustrate the marked difference in Ruthenium Red staining of the two electron-dense leaflets of BZ2. Arrow indicates the proximal portion of the side wall of a cellular compartment boundary. ×53,000.
Volvox extracellular matrix
but with a considerably steeper and higher cone, and with much less material between the two leaflets of BZ2 (Burr & McCracken, 1973). In *V. aureus* (section Janetosphaera) the cone of FZ3a differs from those of the preceding species by being markedly broader and shorter, and by not being lined by an obvious and complete continuation of BZ2 (Fig. 9). In *V. carteri* f. *nagariensis* (section Merillosphaera) FZ3a is completely lined by BZ2, but it is even broader than in *V. aureus* (yielding a hemispherical profile) and its core is less homogeneous than in the preceding species (Fig. 10). In sharp contrast to all the foregoing species, *V. dissipatrix* (section Copelandosphaera) lacks FZ3a entirely (Fig. 8).

Distinctive as the above differences in FZ3a structure are, they clearly do not constitute diagnostic characters for the four different sections of the genus, as was demonstrated by examination of a fifth type, *V. carteri* f. *weismannia*. Unlike forma *nagariensis*, forma *weismannia* does not have a pronounced flagellar hillock; like *V. dissipatrix* it is devoid of such a structure (Figs 13, 14). Although f. *weismannia* and f. *nagariensis* are not interfertile (Starr, 1970a), they are included in the same species by current practice. Whether or not they really are conspecific, it is clear that they are both members of the section Merillosphaera, share many morphological features, and are closely related. Thus the striking difference between them (and the similarity of f. *weismannia* to *V. dissipatrix*) in regard to this particular feature suggests that some details of ECM architecture must be relatively labile phylogenetically, and indicates clearly that considerable caution will be required in using them as taxonomic criteria.

FZ3b, the flagellar tunnel, shows somewhat less variability than FZ3a in the species studied. In *V. globator*, *aureus* and *carteri*, the primary component of FZ3b is a cylinder (of rather different length in the different species) of finely granular material that runs from the base of the flagellum to the base of FZ3a. In *V. aureus*, FZ3b appears to be continuous with the core material of FZ3a (Fig. 9); in *V. globator* such continuity is somewhat more dubious (Fig. 7); and in *V. carteri* f. *nagariensis* it is clear that the two structures are morphologically distinct (Fig. 10). (In the latter case, however, the core material of FZ3a does extend part way down around the outside of the FZ3b cylinder.) As with FZ3a, *V. dissipatrix* exhibits the greatest deviation from the common theme. In it, the outer portion of the flagellar tunnel appears to be lined with continuations of BZ2, different components of which appear to extend to different depths into the tunnel region, and only the deeper portion of the tunnel appears to contain an FZ2b cylinder resembling that seen in the other species (Fig. 8).

The boundary zone. Because some version of BZ2 (the tripartite layer) is present in all Volvocales examined to date, we have used it as a reference point in numbering the components of the boundary zone, and will therefore consider it first. Although always present in some form, BZ2 is not invariant in appearance, even within the genus *Volvox*. In thin-sectioned specimens this region typically appears as two electron-dense layers separated by an electron-lucid layer of constant thickness. This appearance is exhibited with diagrammatic clarity by *V. dissipatrix* (Fig. 8), and almost as clearly by *V. carteri* (Figs 10, 13, 15). (Fig. 15 also illustrates that the inner
leaflet stains more intensely with Ruthenium Red than the outer one does.) In *V. globator* and *V. aureus*, however, BZ2 is modified in appearance. In the former species, the outer BZ2 leaflet has a velvety appearance, with densely packed, very fine filaments of uniform length, aligned perpendicular to the surface (Fig. 7). In the latter species, BZ2 has a five-part appearance: three electron-dense layers separated by two electron-lucid layers (Fig. 9). Although these differences might reflect intrinsic variations in BZ2, it seems more likely that they are simply modifications in appearance caused by the BZ1 components applied to the outer leaflet. Additional subdivisions of the BZ2 region have been proposed for *Chlamydomonas* (Roberts, 1974; Goodenough & Heuser, 1985), but until more detailed analysis has been performed, subdivision of BZ2 in these species of *Volvox* appears premature.

BZ1, the outer portion of the boundary zone, is an almost as distinctive species character as FZ3a. Of the species we have examined, *V. globator* exhibits an as extensive and complex BZ1 region as any. In addition to the velvet on the outer face of BZ2 (which is probably part of BZ1), long, coarse, electron-dense filaments emanate from near the surface of BZ2 and intertwine extensively to cover the entire spheroid with a deep-tangled brushwork. Because these main BZ1 filaments are woven together at their peripheral ends to form a coherent surface network, and because the brushwork rises to even greater heights around the flagella, BZ1 has a tentlike configuration in periflagellar regions (Figs 5, 7). The main BZ1 brushwork of *V. carteri* f. *weismannia* resembles that of *V. globator*, but because the filaments are less coarse and are less abundant toward the periphery, the coherent surface network stands out even more clearly (Fig. 14). The fact that Ruthenium Red is preferentially deposited at the periphery of BZ1 (Fig. 13, small arrows) suggests that the surface network may represent some additional component(s) that interconnects the peripheral ends of the BZ1 filaments. The two forms of *V. carteri* are as different in their BZ1 as in their FZ3a architecture: the BZ1 of forma *nagariensis* is as sparse as that of forma *weismannia* is extensive—consisting of only an occasional clump of short, dense filaments (Fig. 10). (This difference is not an artifact of cultivation or preparative procedures, as it was observed in specimens grown side-by-side and prepared in parallel for microscopy.) In BZ1 structure, *V. aureus* and *V. dissipatrix* lie between the extremes seen in *V. carteri*; both have a modest layer of short, coarse, intertangled filaments that exhibit little additional organization (Figs 8, 9).

A third BZ filament network, internal to BZ2 and distinguishable in texture from the network constituting the cellular envelopes and boundaries, is designated BZ3. In *V. globator* BZ3 constitutes a relatively thick feltwork of distinct filaments (Fig. 7). In *V. carteri* the BZ3 layer and its component filaments are both thinner (Figs 10, 14). In *V. aureus* both the layer and the component filaments are extremely thin, but the separation from the filaments of the cellular zone is more clear cut than in the other species (Fig. 9). In *V. dissipatrix* the interface between BZ3 and CZ3 is frequently difficult to discern, because the filaments are very similar in size and packing density, and only occasionally does a space appear between the two zones. But the filaments of BZ3 are preferentially aligned perpendicular to the surface, and those of the outer portion of CZ3 are preferentially aligned parallel to it (Fig. 8).
The cellular zone. In unicellular Volvocales obviously no meaningful distinction can be drawn between ECM components that surround individual cells (CZ) and those that surround the organism as a whole (BZ), since cell and organism are synonymous. But in multicellular forms the distinction is real, and understanding the genetic basis for it will be important for understanding the origins of multicellularity in the order. As will be seen below, in certain species of *Volvox* two cellular layers are present and subdivision of CZ may be considered in such cases.

The archetype of *Volvox* CZ organization is exhibited by *V. globator* (Figs 5, 24). A thin, coherent network of ECM filaments (CZ1) is closely applied to the plasma-lemma. An equally coherent network of filaments (CZ3) underlies BZ3 to form the roof, runs midway between neighbouring cells to form the walls, and then runs below all cells to form the floors of the cellular compartments. The chambers enclosed by CZ3 are filled with a much looser meshwork of filaments (CZ2), some of which appear to be continuous with those of CZ1 or CZ3. Gonidia of *V. globator* originate in the same layer as the somatic cells and originally have the same CZ architecture; but as the embryos derived from the gonidia expand, they first fill their cellular compartments (so that CZ1 and CZ3 merge and CZ2 is obliterated) and then bulge into the deeper regions of the spheroid (Figs 4, 24). Both forms of *V. carteri* (and all of their relatives in the section Merrillosphaera) differ from *V. globator* in one important regard that affects CZ organization: because *V. carteri* gonidia are much more...
larger than somatic cells from the time they are formed during embryogenesis, and withdraw from the somatic cell layer very early, they lie internal to the somatic cells throughout the period of ECM deposition, and possess much more clearly defined CZ1, CZ2 and CZ3 regions throughout life (Figs 16, 24). Otherwise CZ architecture is basically similar in *V. carteri* and *V. globator*, except that the ratio of cell compartment volume to cell volume is larger in the former (cf. Figs 5, 16).

*V. dissipatrix* exhibits a clear departure from the two preceding species in CZ organization: although the roofs and walls of its cellular compartments are well-
formed, floors are entirely missing (Figs 20, 24). One consequence of the absence of compartment floors is that CZ2 (which has a much more regular filamentous substructure in this than in the other species) extends deep into the interior, well below the termini of the compartment walls (but as will be shown shortly, it does have a defined terminus). *V. aureus* carries the trend to incomplete compartment boundaries even further: in this species, not only the floors, but also the side walls of the compartments are missing! Nevertheless, *V. aureus* possesses a diversity of CZ specializations. CZ1 is present and relatively undistinguished (Fig. 23). In contrast, the structure of CZ3 is unique: instead of forming a series of distinct cellular compartments, it is merely draped from cell to cell across the edges of the intercellular spaces (Figs 21–23), and from its lower edge, midway between neighbouring cells, delicate tongues of very fine filaments descend, perhaps representing vestiges of the compartment walls (Figs 22, 23). An extensive and coherent network of filaments (which we take to be analogous to CZ2 in other species) begins just below the level of the cytoplasmic bridges (Fig. 22) and fills much of the interior of the spheroid (Fig. 21). Finally, as Smith (1944) recognized, *V. aureus* possesses one ECM specialization not seen in any of the other species: filamentous strands radiate at irregular intervals from the edge of DZ1 (near the centre of the spheroid) to the boundary zone (Figs 21, 24). The significance of these radial fibres is obscure.

Because colonial Volvocales possess complete cellular compartments, the incompleteness of the cellular compartments in *V. dissipatrix* and *V. aureus* appear to be a derived, rather than a primitive, trait.

The deep zone. Just as the existence of a distinct CZ distinguishes multicellular from unicellular Volvocales, so the existence of a distinct deep zone (DZ) within the ECM distinguishes the higher from the lower multicellular forms. In small colonial forms such as *Pandorina morum*, cell compartments are conical and abut nearly all the way to the centre of the colony, leaving only a tiny central space that could be considered the forerunner of the DZ (Fulton, 1978^6). In sharp contrast, in *Volvox*, the DZ may constitute more than 90% of the total volume of the spheroid (Fig. 3). (Bell (1985) has recently discussed the possible physiological, ecological and evolutionary significance of the development of the DZ (which he refers to as the ‘colony lumen’) at some length.)

In *V. globator*, a clear-cut demarcation between CZ and DZ occurs in the form of a band of intensely staining fibres (DZ1) lying just below CZ (Figs 3, 24). Although DZ1 looks quite coherent in the young spheroid, as embryos enlarge toward the interior of the spheroid they appear merely to push the DZ1 fibres aside and expand into DZ2 (Fig. 4). In *V. carteri*, the interface between CZ and DZ is less intensely stained, but DZ1 is clearly present as a band of concentrically aligned filaments lying just internal to the gonidia (Fig. 17). Surprisingly, perhaps (in view of the fact that its cellular compartments lack floors), *V. dissipatrix* exhibits one of the most distinct and complete separations between CZ and BZ: here, a thick coherent band of intensely stained DZ1 fibres form a sharp demarcation between the two regions that is clear even at very low magnification (Figs 19, 24). Unlike the situation in *V. globator*, the *V. dissipatrix* embryos distort (but never penetrate) the DZ1 layer
when they enlarge into the interior (Fig. 19). Thus it appears that in both *V. carteri* and *V. dissipatrix* the limit of the cellular zone is further from the surface of the spheroid than it is in *V. globator*. *V. aureus* carries this trend to a startling extreme: the filamentous DZ1 layer separating CZ from DZ lies very near the centre of the spheroid (Fig. 21). As a result, whereas the DZ constitutes 90%, or more, of the total volume of the spheroid in other species, it constitutes less than 10% of the volume of *V. aureus*. The differences in CZ/DZ organization in the four sections of the genus are summarized diagrammatically in Fig. 24.

**CONCLUSION**

The intricate variations of ECM architecture within and among *Volvox* species are truly impressive. Unravelling the mechanisms by which *Volvox* cells orchestrate the precise construction of all these defined structures at considerable distances from their own cellular boundaries will be a challenging but fascinating task. Unravelling the genetic bases for the phylogeny of such a complex system of extracellular architecture, from its relatively simple origins in the unicellular Volvocales, will be at least as challenging. But, as outlined in the Introduction, many of the tools that will be required for studies of both ECM ontogeny and phylogeny are under development, and the prospects for making considerable progress in solving these puzzles appear at present to be at least as great in the case of the Volvocales as in the case of either the higher plants or animals. We are hopeful that the present study will contribute to future work, both by indicating the magnitude of the ECM specializations within the Volvocales that are ultimately to be explained, and by providing a coherent set of terms that will be useful for dissecting and discussing the system.

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