STUDIES OF SPERMATOGENESIS IN THE HEPATICAE

I. Ultrastructure of the *Vierergruppe* in *Marchantia*

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ABSTRACT

Spermatids of the liverwort *Marchantia polymorpha* were shown by analysis of thin-sections to contain a four-layered organelle, the *Vierergruppe*, lying between the flagellar bases above and a subjacent mitochondrial body. The uppermost layer consists of parallel tubules structurally similar to those of the axoneme. Some of the tubules extend beyond the four-layered region to form a long ribbon-like appendage. The second layer consists of low, vertical, membranous fins connected by numerous short links to the tubules above. The third layer, limited above and below by two horizontal membranes, consists of vertical partitions, apparently continuous with the fins. The fourth and lowermost layer consists of narrow parallel tubules aligned beneath alternate partitions. The long axes of the lower three layers are parallel to each other and lie at an angle of 48° to the tubule axis of the uppermost stratum. A graphic representation of a three-dimensional scale model interpreting organelle structure is given. The *Vierergruppe* supersedes *Dreiergruppe* interpretation in most if not all of the bryophytes studied to date.

INTRODUCTION

The discovery of a new cytoplasmic organelle in spermatids of four liverworts was reported in 1959 by Heitz (2). Working with *Marchantia polymorpha* and other species, he described a stratified structure which, together with a subjacent chondriosome-like body, lay immediately beneath the two flagellar bases. The new organelle comprised three layers and was, accordingly, designated *die Dreiergruppe*. The upper layer was provisionally called a double spiral because it appeared to consist of double wave lines (*doppelte Wellenlinie*); the middle layer was cross- or diagonally striped; the lower stratum was indistinct, sometimes appearing as lines or rings. Heitz tentatively interpreted the *Dreiergruppe* as a longitudinal section of a flagellar base, the three components of the group corresponding to flagellum fibrils. After further study, Heitz announced the existence of the *Dreiergruppe* in spermatids of mosses as well as liverworts (3).

He again showed the *Dreiergruppe* as a tripartite structure which is usually associated with an underlying chondriosomal body. In that report, he also pointed out that he could find no clues about the connection between *Dreiergruppe* and flagellum, nor could he offer a structural interpretation of the new organelle. Recently, a tentative model interpreting *Dreiergruppe* structure was offered by Paolillo who investigated androcytes of the moss *Polytrichum* (9). He showed conclusively that the upper layer was a long band of parallel tubules. He interpreted this band as a structure identical with the so called filamentous appendage observed in various bryophyte spermatozoids by Satô (12, 13) and as analogous with the tubular elements or "roots" described for other motile plant cells (6, 7). Paolillo further suggested that the lightly stained, upright stripes of the middle layer and the light circles of the lower layer were,

respectively, vertical columns and horizontal rods of electron-transparent material, each embedded in a dark-stained matrix.

The present writers reexamined *Dreiergruppe* structure in *Marchantia* as part of a broad investigation of spermatogenesis and antheridial development in selected liverworts. Our early micrographs clearly indicated several discrepant departures from the published reports and emphasized the need for a detailed study. The following account focuses on the ultrastructural characteristics of the organelle's component layers and their interrelationships. A second part dealing with gross aspects of organelle morphology and with the structural and functional relationships between this and other spermatid organelles is in preparation.

MATERIALS AND METHODS

Male heads of *Marchantia polymorpha* L. were dissected, and whole antheridia in various stages of development were obtained. The antheridia were fixed in buffered glutaraldehyde and postfixed in osmium tetroxide (4). Dehydration was carried out in a graded ethanol-water series as follows: 10 min in each of 5% steps from 5 to 50%, then 10% steps to 100%. After a short graded transfer to propylene oxide, we embedded the material in Araldite-Epon, using Mollenhauer's formula No. 1 (8). Thin sections were cut with a diamond knife, and stained with uranyl acetate (17) and basic lead citrate (11). All specimens were observed with an Hitachi HU-11a electron microscope. The organelle dimensions presented here are based upon calculated averages of five or more measurements.

Our use of the term "membrane" in regard to certain *Vierergruppe* components takes cognizance of the fact that these structures apparently lack the three-layered morphology common to unit membranes. A detailed analysis of the point is beyond the scope of this paper; for the present, the term should be construed as having a more general meaning.

OBSERVATIONS

Structural analysis of the *Dreiergruppe* in our material soon revealed that the organelle comprises four, instead of three distinctly different layers. Since our findings were consistent in this regard, it seemed desirable to use a more appropriate designation which would reflect this consistency. In this report, we have used "*Vierergruppe*" (group of four) as a preferable term, believing that such usage would tend to eliminate inconsistencies and contribute to a smoother

presentation. Rationale and justification for its continued use have been included in the Discussion section.

The *Vierergruppe* of a *Marchantia* spermatid is an elongated structure that lies just beneath the cell surface. Preliminary observations of its gross morphology indicate that it bears superficial resemblance to a curved sewing needle, the eye-containing portion of which is somewhat expanded for a short distance. The main body of the *Vierergruppe* is restricted to the expanded portion and represents the anterior end of both organelle and cell. Directed posteriorly, the shank portion, designated here as *Vierergruppe* appendage, is narrow, ribbon-like, and long, extending halfway or more around the cell. Although the *Vierergruppe*, or VG, is continuous with the *Vierergruppe* appendage, VGA, the former is much more complex in structure. Implicit in Heitz's designation "*Dreiergruppe*" and in the substitute term "*Vierergruppe*" is the characteristic multilayered nature of this organelle. In contrast, the appendage consists of a single stratum and is, in fact, a prolongation of a portion of the *Vierergruppe*'s outermost layer.

The outermost component of the *Vierergruppe*, i.e. that layer closest to the cell surface, is designated as VG_1. It consists of a band of tubules whose long axes are essentially parallel to each other and to the plasma membrane (Fig. 1). The commonest number of VG_1 tubules we have observed in a single cell is 17. Measurements taken from transverse sections show that the individual tubules have an outside diameter of 265 A and an inside diameter of about 140 A. Their center-to-center spacing is 320 A. Transverse sections also reveal that each VG_1 tubule has a keel or longitudinal ridge running along its centerline at bottom (Figs. 2, 3, and 13). The average keel is about 150 A wide and projects down from the tubule about 100 A. Their cross-sectional profiles vary from rectangular or square to tapering. A side view of the keel shows its lower edge to be relatively flat (Fig. 3). In longitudinal or near-longitudinal sections, at least some of the VG_1 tubules can be seen extending beyond the margin of the lower strata of the *Vierergruppe* and the subjacent mitochondrial body (Figs. 4 and 5). These tubules constitute the bandlike appendage which at this stage of development partially encircles the cell. Keels are present on VGA tubules only in those portions of the appendage that are situated directly above the lower strata of the *Vierergruppe*. For
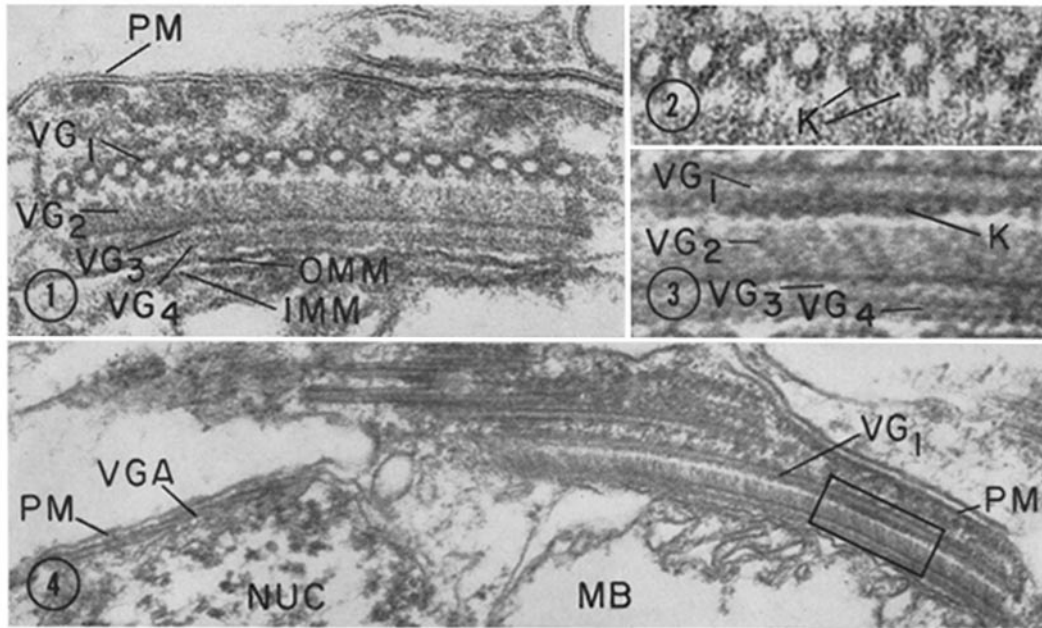
FIGURE 1 Transverse section of the *Vierergruppe* showing four strata (VG$_1$–VG$_4$). The beaded appearance of the VG$_1$ tubule walls is attributed to the subunits of which the walls are composed. × 136,000.

FIGURE 2 Transection of VG$_1$ tubules showing detail of the keels. × 235,000.

FIGURE 3 Enlargement of area outlined in Fig. 4. × 195,000.

FIGURE 4 Longitudinal section of the *Vierergruppe*, the anterior part of which is at right. Five tubules of the appendage appear in longitudinal-oblique section (shown at left). × 56,500.

much of their length, the VGA tubules lie sandwiched between the plasma membrane and the nucleus (Fig. 6). The number of tubules constituting the VGA is commonly about six. Both transverse and tangential sections taken at various positions along the length of the appendage indicate a relative constancy in intertubule spacing. Some of the tangential sections suggest the existence of cross-links between adjacent VGA tubules (Figs. 7 and 8). There is further suggested in Fig. 8 an oblique orientation of cross-links which would, in effect, align them in diagonal rows with respect to the long axis of the VGA. Although we consider Figs. 7 and 8 insufficient evidence to warrant an unequivocal statement concerning cross-links, we do believe them to be strongly indicative of such links. Other observations that must for the present be considered as somewhat speculative concern the substructure of the tubules of VG$_1$ and VGA. Some indication of the particulate nature of tubule construction is given in Figs. 1 and 6. Our measurements from transverse sections seem to indicate that there are about 10 subunits per tubule circumference. The subunits are approximately 60–65 A in diameter and have a similar center-
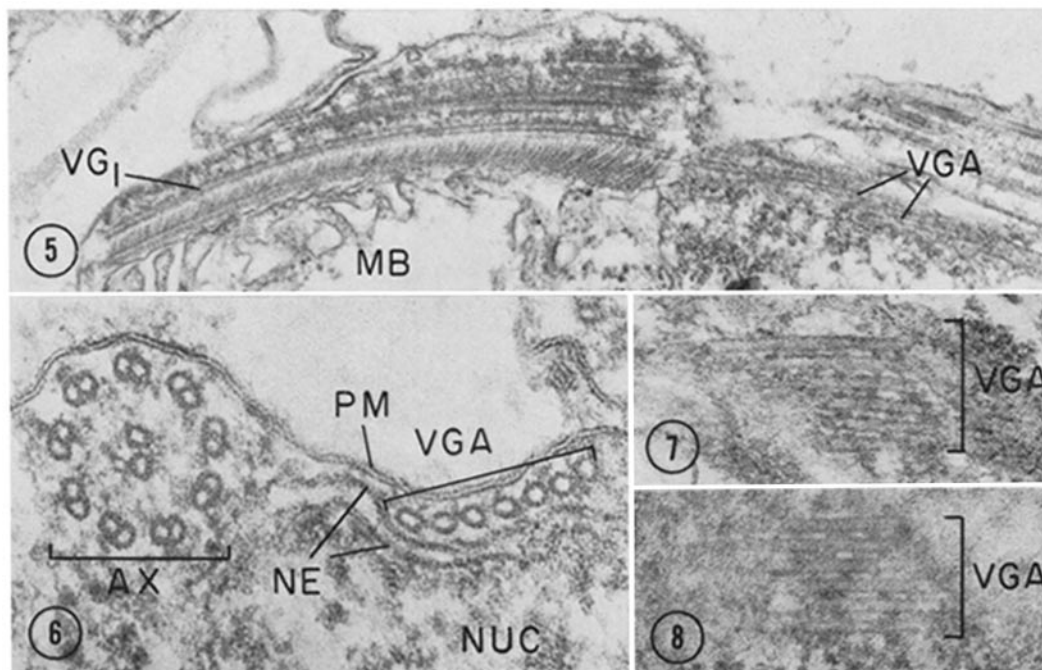
FIGURE 5 Longisection showing VG₁ tubules continuing into the appendage. Anterior of organelle is at left. × 49,000.

FIGURE 6 Transverse section of *Vierergruppe* appendage and axoneme showing their proximity to the cell surface. Beaded appearance of subunits is particularly clear in central tubules of axoneme. × 136,000.

FIGURES 7 and 8 Tangential sections of *Vierergruppe* appendage. Each micrograph suggests the existence of cross-links between adjacent tubules. × 80,500 and 87,000, respectively.

to-center spacing. The keel also appears composed of subunits like those in the tubule proper (Fig. 2).

Transverse and longitudinal sections of the *Vierergruppe* generally show three distinct layers of unequal thickness and density lying beneath the VG_1 and separated from it by a thin, lightly stained zone (cf. Figs. 1 and 4). While these same sections show VG_1 tubules in sharp detail, they present few, if any, structural details of the underlying strata. However, certain sections cut diagonally, i.e. at right angles to a line nearly midway between the transverse and longitudinal planes, reveal a surprisingly elaborate morphology (Figs. 9 and 10). The upper and middle of the underlying layers are interpreted as uniform series of dark-stained membranes embedded in a nearly electron-transparent matrix. The upper stratum, designated VG_2, comprises numerous, vertical finlike membranes joined at their lower edges to a horizontally oriented membrane. The

fins are 70 A thick and are aligned on parallel axes 140 A apart. They extend 390 A above the center of the horizontal membrane. Beneath lies another horizontal membrane that is parallel to the one above. These two horizontal membranes, which henceforth will be referred to as upper and lower horizontal membranes, have a center-to-center vertical separation of 150 A, and are each 35 A thick. Together they delimit the vertical extent of the middle underlying layer, or VG_3. The VG_3 layer is compartmented by a series of vertical partitions, each 35 A thick—half the thickness of those in VG_2—and each centered on the same axes as the VG_2 fins.

Confirmation of the angled orientation of VG_2 relative to VG_1 and of the interpretation of VG_2 vertical components as finlike structures is obtained when these layers are seen in tangential section (Figs. 11 and 12). The short, dark vertical stripes of VG_2 seen in diagonal section now appear as
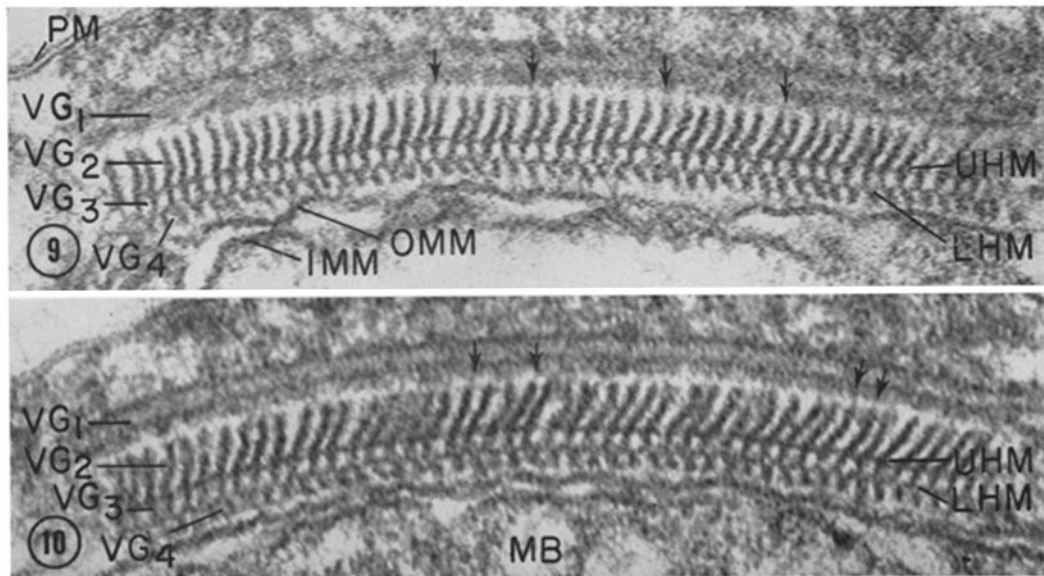
FIGURES 9 and 10 Diagonal sections of the organelle showing the lower three strata in cross-section. Connections between VG₁ keels and VG₂ fins are indicated by arrows. × 171,000 and 180,500, respectively.

long, parallel lines and give the stratum a distinctive hatched appearance. The apparent unequal length of these vertical fins is determined by the curved margin of the *Vierergruppe* and by its surface curvature relative to the tangential plane of section. The conspicuous horizontal lines immediately to the left of the fins in Figs. 11 and 12 represent VG₁ tubules. The superimposed long axes of VG₁ and VG₂ intersect to form a 48° angle. These two strata are, in fact, connected to each other in a manner that stabilizes the spatial relationships between them. Traversing the narrow, virtually unstained horizontal zone that separates VG₁ from VG₂ are thin vertical strips of very lightly stained material (Figs. 9, 10, and 13). Their low contrast makes them extremely difficult to demonstrate. The connections extend approximately 50 A from the upper edge of the VG₂ fins to the VG₁ keel above and are oriented parallel to the fins. Their length is assumed to be equal to the linear extent of a VG₂ fin's intersection across a VG₁ keel; that is, approximately 200 A. The thickness of these connections is estimated to be 20–35 A. We have not yet been able to determine this dimension with reasonable certainty.

The lowermost *Vierergruppe* stratum, or VG₄, consists of a single layer of small tubules appressed

to the lower horizontal membrane. Since their axes are aligned parallel to those of the VG₂ and VG₃ vertical components, the tubules appear in diagonal section as small circles (Fig. 14). In other planes of section they appear as ellipses (Fig. 3) or as rather indistinct parallel lines. Diagonal sections also show them to resemble the upright curves of closely spaced parentheses, in which case they are interpreted as having been ruptured along their centerlines at bottom (Figs. 9 and 10). The VG₄ tubules are aligned beneath alternate VG₃ partitions and have, therefore, a center-to-center spacing of 280 A. Measurements indicate their outside diameters to be about 175 A and inside diameters about 65 A. Unfortunately, we were unable to obtain more than sketchy, inconclusive information concerning the details of tubule structure. Our material seems to indicate seven to eight subunits per tubule circumference. The difference between outside and inside tubule diameters suggests that the subunits are about 55 A wide. The center of each tubule lies about 75 A below the middle of the lower horizontal membrane. A line drawing depicting a three-dimensional interpretation of the *Vierergruppe*'s multilayered structure is presented in Fig. 15. Over-all thickness of the *Vierergruppe* is about 1100 A.
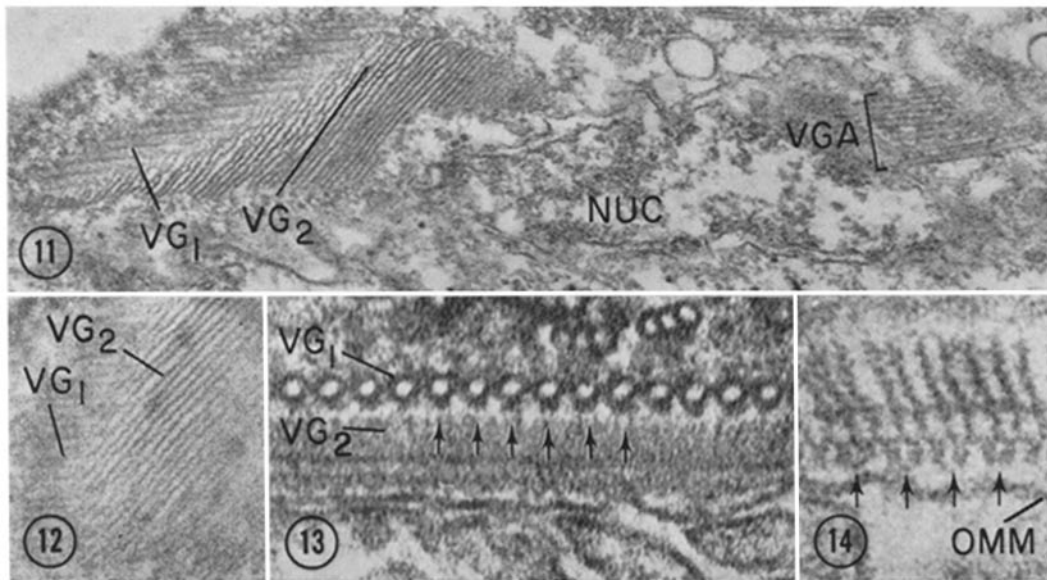
FIGURE 11   Tangential section of organelle showing intersection angle of VG$_1$ tubules and VG$_2$ fins. Superposed above the nucleus at right is a portion of the appendage. × 55,000.

FIGURE 12   Tangential view of VG$_2$ fins. Note absence of cross-connections between adjacent fins. × 86,500.

FIGURE 13   Transection of organelle. Arrows indicate connections between VG$_1$ keels and VG$_2$ fins. × 146,000.

FIGURE 14   Diagonal section of *Vierergruppe*. Arrows indicate four of the VG$_4$ tubules aligned on alternate VG$_3$ partition axes. × 213,000.

DISCUSSION

Analysis of thin sections cut in various planes shows that VG$_1$ consists of a layer of parallel tubules, a few of which extend considerably beyond the margin of the lower strata to form a ribbon-like appendage. This general configuration is similar to that described for *Polytrichum* androcytes by Paolillo (9) and it thus supports his account which described for the first time the unity of appendage and organelle. Our preliminary comparison of VGA structure with Satô's description of "filamentous appendage" in eight liverworts including *Marchantia* (12, 13) and with Manton's account of the "fibrous band" in *Sphagnum* (6) has shown essential similarity among them. Unfortunately, few quantitative data regarding the component tubules are available. Satô (12) found the outside diameter of filamentous appendage tubules to be 20 mμ, which coincides with the lower limit of the 200–250 A

range given by Heitz (2). The 265-A diameter of VG$_1$ tubules in the present study, although just above the upper limit given by Heitz for the same species, is considerably above the 150-A diameter of the tubules in *Polytrichum* (9). Whether other mosses have equally narrow tubules is not presently known. Heitz included the mosses *Splachnum* and *Physcomitrium* in his later study, but gave no specific measurements for either. A striking similarity does exist between the VG$_1$ tubules and those constituting the axoneme. Measurements show that their respective diameters are virtually identical, a condition comparable to that described by Silveira and Porter for cortical microtubules and flagellar filaments in flatworm spermatozoids (16). Analysis of micrographs showing the tubules in rather exact transection indicates strongly that substructures of VG$_1$ and axoneme tubules are also the same. The tubule walls in our material appear to comprise ten subunits, thus matching in
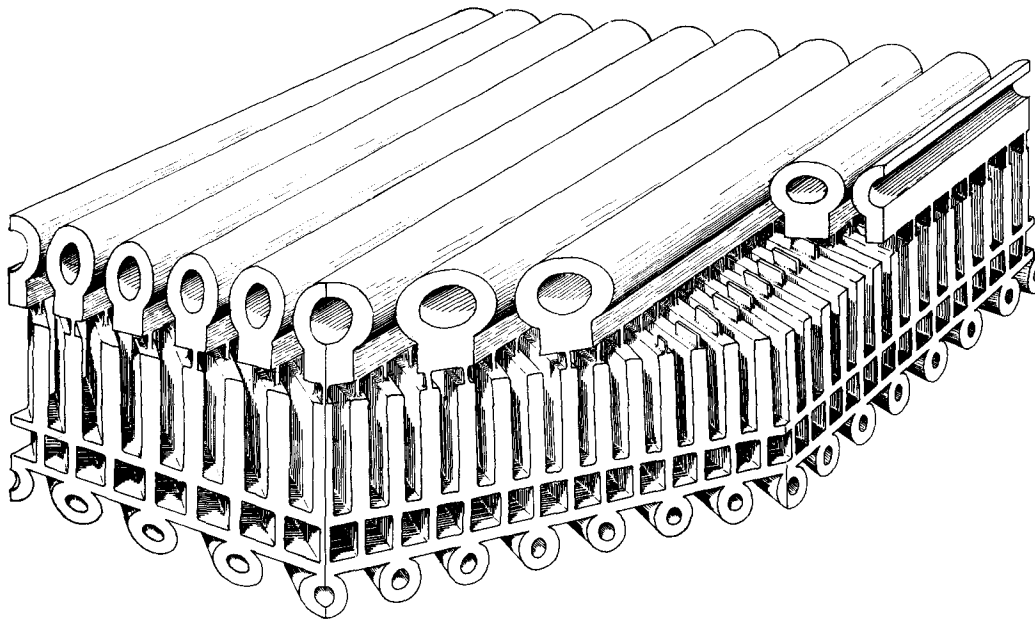
Figure 15   A three-dimensional, interpretive line drawing of *Vierergruppe* ultrastructure.

number the findings of André and Thiéry (1), who worked with human sperm cells, and of Pease (10) who studied sperm tails of the rat. Those investigators, however, reported subunit diameters of 35-40 A, whereas the subunit diameters in *Marchantia* are 60–65 A. It is questionable whether this appreciable difference might be attributed to different materials and techniques. In both of those studies, negatively stained preparations were used; our procedure was basically similar to the sectioning technique of Ledbetter and Porter (4). Interestingly, our findings are closely comparable to the 70-A diameter subunits found in cytoplasmic microtubules by Ledbetter and Porter (4, 5). Another similarity between axoneme and VG_1 tubules concerns their common response to certain experimental procedures. Satô (13, 14) subjected mature spermatozoids of *Marchantia, Conocephalum*, and other liverworts to hot water treatments and found that only the fibrils of flagella and "filamentous appendage" remained intact after nucleus and cytoplasm were dispersed. Further, the fibrillar components of the appendage had undergone only slight lateral separation. Satô (14) obtained similar results by disrupting spermatozoids with ultrasonic waves, except that there was little or no separation of fibrils forming the appendage (cf. his Figs. 15 and 16). The relatively

uniform spacing of about 50 A between adjacent tubules of the *Vierergruppe* appendage is most probably maintained by cross-linkage. The resistance to tubule separation shown by Satô's experiments may be interpreted as indirect evidence supporting the existence of cross-links. Further support, albeit inconclusive, is found in the tangential section micrographs included in this paper.

The presence of four different component layers in the *Marchantia Vierergruppe* is clearly demonstrated in diagonal sections. This orientation is at right angles to the long axes of the three lower layers and reveals probably more structural information than any other single plane of section. The diagonal aspect shows the VG_2 resemblance to a uniform layer of alternate dark and light stripes similar to the comparably striped layers shown by Heitz and Paolillo in other liverworts and mosses. The present interpretation of these dark stripes as long vertical fins is completely substantiated from tangential sections. In fact, tangential sections used in conjunction with the other planes of section constitute the only fully acceptable proof of such an interpretation. Comparison of diagonal and tangential sections (e.g. Figs. 9 and 12) shows clearly that the VG_2 fins are completely separated from each other by an

electron-transparent material. The dark-stained fins and the absence of upright partitions between them preclude the interpretation of VG_2 according to the model proposed by Paolillo for *Polytrichum* (9). He interpreted the striped layer (DG_2) as orderly rows of cylindrical columns of electron-transparent substance in an "electron-dense" matrix. As previously indicated, the connections that traverse the narrow light zone between VG_1 and VG_2 are hard to distinguish because of their low contrast against that unstained background. However, they have been seen in abundance in all but tangentially sectioned material. Moreover, they may be seen in micrographs of other species. For example, keels and connections are visible in some of the pictures in Heitz's 1959 paper [reference 2; see Figs. 5 and 9 (*Marchantia*), Fig. 13 (*Sphaerocarpus*), and Fig. 15 (*Pellia*)] and also in his 1960 paper [reference 3; see Fig. 6 (*Splachnum*)]. Paolillo (9) also indicated the existence of connections of undetermined nature in *Polytrichum* (cf. his Figs. 8, 9, 10, 17).

The VG_3 layer is limited vertically by the upper and lower horizontal membranes. Purely on structural grounds, the two membranes should at least contribute substantially to the support of VG_2 fins since the fins and the VG_3 partitions appear to be confluent across the membranes. Moreover, the horizontal membranes appear to have an integral part in maintaining the spacing of the partitions and fins. The compartmented construction that characterizes VG_3 together with the fins of VG_2 provide extensive membrane surface area in a rather restricted region. The significance of the difference in thickness between the membranous components of VG_2 and VG_3 is doubtful. The thinner VG_3 components do stain more lightly, with the result that this layer is somewhat more readily identifiable in micrographs of lower magnifications. In his description of the *Dreiergruppe*, Heitz (2) said that it was composed of three structures separated by narrow, lighter bands. Examination of his pictures shows that the lower of the light bands is, indeed, the layer identified here as VG_3; for example, his Fig. 14 (*Sphaerocarpus*) and his Fig. 18 (*Pellia*) clearly show the narrow lighter band traversed by regularly spaced vertical partitions. Fig. 15 of Paolillo's paper is suggestive of the same thing (9).

The lowermost stratum of the *Vierergruppe* consists of parallel tubules that are smaller and much more delicate than those of VG_1. Frequently, the

VG_4 tubules appear to contact the outer membrane of the subjacent mitochondrial body. When there is a relatively conspicuous separation between these two, as shown in Fig. 9, the tubules commonly have the "parentheses" appearance. This configuration is interpreted as an artifact most probably associated with fixation. There is little doubt that the VG_4 tubules constitute the third (lowermost) layer of the *Dreiergruppe*. The term "*Dreiergruppe*" as proposed by Heitz implicitly connotes a characteristic multilayered morphology and, at the same time, explicitly denotes three constituent layers. The data presented here, however, show unequivocally that in *Marchantia* the organelle comprises four layers. We suggest that *Vierergruppe* structure can be recognized also in each of the three liverworts and the one moss illustrated in Heitz's papers. For evidence in support of this view, the reader is referred to Fig. 9 (*Marchantia*), Fig. 14 (*Sphaerocarpus*), and Fig. 18 (*Pellia*) in Heitz's first report (2) and to Fig. 6 (*Splachnum*) in his second (3). If one wished to conserve the original designation "*Dreiergruppe*," he probably would, on the basis of certain morphological similarities, identify VG_2 and VG_3 as DG_{2a} and DG_{2b}, respectively. Such usage would carry a concomitant implication that the two strata are related in some special way. But the relationships between these strata are presently enigmatic, or at best highly speculative, because composition, ontogeny, function, and ultimate disposition of the strata are essentially unknown. From the foregoing, it seems reasonable to recommend the more appropriate "*Vierergruppe*" as a substitute term for the four-layered structure.

Presently there is not enough evidence to warrant the conclusion that all such organelles have *Vierergruppe* structure. From the published micrographs, one cannot tell with certainty whether the organelle in *Polytrichum* has three or four layers. However, the similarities between DG_1 and DG_2 of that moss and VG_1 and VG_2 of our material strongly suggest that *Polytrichum* may be more correctly interpreted according to the model presented here (Fig. 15). A reexamination of *Polytrichum* that includes tangential sections of the organelle would determine whether the second layer consists of "cylindrical columns" or long fins and whether the axes of the top layer superimposed over those of the lower strata formed a 90° angle or one about half that value, as shown in our model. A detailed study of the *Polytrichum* organelle

would also ascertain the "rod" or tubule nature of the lowermost layer. Several problems emphasize the need for a detailed developmental study of the *Vierergruppe*. At present, it is not known when this organelle first appears in the spermatogenic process. It has occurred in all the spermatids we have examined thus far, including very young ones. This suggests the possibility that organelle ontogeny may be initiated after the penultimate rather than after the ultimate cell reproduction, that is, in a spermatid mother cell. Interestingly, Satô (15) mentioned no such organelle in his study of spermatid structure in *Conocephalum conicum*, a closely related form, although a *Vierergruppe* has recently been found by one of us (G. L. K.) in the spermatid of *Anthoceros laevis* in a

preliminary study of spermatogenesis in the distantly related hornworts. An ontogenetic study should tell whether the VG strata undergo a sequential mode of development so that a *Vierergruppe* might appear tripartite or *Dreiergruppe*-like in an earlier stage. Such an investigation might also yield information concerning possible homologies of the *Vierergruppe* strata.

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