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Development of *Dentalium* Following Removal of D-Quadrant Blastomeres at Successive Cleavage Stages

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Summary. Each primary micromere and macromere of the D-quadrant of *Dentalium* was deleted, through the mesentoblast stage, to investigate the way in which the polar lobe cytoplasm exerts its influence on development. −D and −1D embryos form an apical tuft but no posttrochal structures. −2D embryos form an apical tuft and a reduced posttrochal region without a shell. −3D and −4D are externally similar to control embryos. −1d embryos and −1c embryos have an apical tuft with a reduced number of cilia. Embryos in which both 1c and 1d are deleted lack the apical tuft. −2d embryos lack shell and most other posstrochal structures. −3d and −4d embryos appear externally equivalent to controls.

The polar lobe cytoplasm exerts its influence sequentially, and as in *Ilyanassa* the maximal effect is at the third quartet stage.

Key words: Mollusca – Embryology experimental – Development analysis – *Dentalium* – Invertebrate development.

Introduction

Wilson's (1904) study of the polar lobes influence on development has been of paramount importance in the conceptual growth of the field of embryology. It is remarkable that his experiments with *Dentalium* were neither confirmed nor extended for more than 60 years when Verdonk (1968a, b), Timmermans et al. (1970), Geilenkirchen et al. (1970) and Verdonk et al. (1971) undertook these investigations. Recently Van Dongen and Geilenkirchen (1974, 1975) and Van Dongen (1976a, b; 1977) have studied the cell lineage of both normal and lobeless embryos while Reverberi (1970, 1971, 1972) has considered the fine structure and cytochemistry of oocytes and eggs of *Dentalium*.

Through the years it has become apparent that those embryos considered to be classic 'mosaic' embryos such as *Dentalium, Ilyanassa,* and *Patella* (see Clement 1971; Cather 1971; Van den Biggelaar 1977) have a complex array

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of cell and tissue interactions. Morrill et al. (1973) have shown essentially complete regulation after deletion of one blastomere at the 2-cell stage of *Lymnaea*. Verdonk and Cather (1973), Cather and Verdonk (1974), Dohmen and Verdonk (1974, 1979) and Cather et al. (1976) have shown that lobe dependent features require the presence of the vesicles of the vegetal body in *Bithynia* which presumedly are distributed to the C macromere as well as to D.

With these changes in viewpoint, we felt it to be necessary to investigate the way in which the polar lobe material of the D quadrant exerts its influence in *Dentalium,* as Clement (1962) did with *Ilyanassa,* by sequentially deleting the blastomeres of the D quadrant.

Materials and Methods

The work for this paper was done at the Station Biologique de Roscoff, on the north coast of Brittany, France, with the eggs and embryos probably of *Dentalium vulgare* da Costa. These specimens although more like *D. vulgare* have intergrading characters with *D. entalis* L. Voucher specimens # 243607 are in the collection of the Mollusk Division, Museum of Zoology, The University of Michigan. Animals dredged from depths just below the intertidal to 30 m were brought into the laboratory and kept in a 10 cm layer of sand in plastic dishpans. A gentle stream of fresh sea water flowed into the containers so that the sand was not disturbed. In this situation, animals remain in excellent condition for at least 6 weeks and probably for considerably longer. Animals were isolated in finger bowls of filtered sea water at room temperature when gametes were required. Although they were isolated at different periods throughout the day, most spawned between 11.00 and 14.00 h. The *Dentalium* were therefore routinely isolated at 9.30.

After the animals spawned, the eggs were rinsed in Millipore filtered antibiotic sea water (ASW) containing 200 μg/ml of streptomycin sulfate and 100 units/ml penicillin G, and fertilized with a drop of sperm suspension directly from a finger bowl containing a male. The percentage of eggs fertilized decreased rapidly after spawning and the percentage of polyspermic eggs and eggs with abnormal cleavages increased. The optimal period for fertilization is within the first half hour after spawning.

Operations were done with hand pulled glass needles. Cells to be deleted had to be cut clear across the surface and cleaned out from their position on the embryo. Simple penetration of the cell by a needle does not lead to cytolysis.

Embryos were reared in Boveri dishes in ASW. Only 3–4 operated embryos or about a dozen control embryos were kept in a single dish.

Each embryo was studied at several stages in the first day or two, to determine its general characteristics and the appearance and nature of its ciliation. Embryos were fixed when it appeared that development was not progressing but while the embryos were still motile and in good condition. Other embryos were observed until they died. Embryos were fixed in Da Fano's fixative, stained with A_gNO_3 according to the method of Van Dongen and Geilenkirchen (1974), and prepared as whole mounts. Other embryos, fixed in Bouin's fixative were treated according to usual histological methods. Serial sections of 5–7 μm were stained with iron hematoxylin and eosin.

Results

Control and Lobeless Embryos

Normal development of *Dentalium vulgare* is in complete agreement with the description of Kowalevsky (1883) of the development of *Dentalium dentale* except

Table 1. Summary of operations

Cell(s) deleted	Cells remaining	Age (hs) (Number of embryos)	General results	
			+ Present Apical tuft	− Absent Posttrochal region
− 1st polar lobe	−	48 (16)	−	−
− 2nd polar lobe	−	48 (20)	+	−
− CD	AB	48 (27)	−	−
− AB	CD	48 (15) 96 (6)	Displaced + Some structures	
− D	ABC	48 (32) 72 (9)	+	−
− 1D	ABC + 1d	48 (9) 72 (39)	+	−
− 2D	ABC + 1d + 2d	48 (20) 72 (8)	+	Some structures
− 3D	ABC + 1d + 2d + 3d	48 (9) 72 (9) 96 (2)	+	Normal
− 4D	ABC + 1d + 2d + 3d + 4d	48 (2) 72 (4) 96 (3)	+	Normal
− 1d	ABC + 1D	48 (16)	Reduction of cilia	Normal
− 1c	ABD + 1C	48 (6)	Reduction of cilia	Normal
− 1c1d	AB + 1C + 1D	48 (3)	−	Normal
− 2d	ABC + 2D + 1d	48 (9) 72 (25)	+	Some structures, *no shell*
− 3d	ABC + 3D + 1d + 2d	48 (2) 72 (8) 96 (4)	+	With foot, shell, etc, variable
− 4d	ABC + 4D + 1d + 2d + 3d	48 (2) 72 (3) 96 (3)	+	Normal

that the rate of development of the Mediterranean species is more rapid. The lower temperatures at Roscoff may account for the differences.

Although Van Dongen and Geilenkirchen (1975) and Van Dongen (1976a, b) had done extensive studies of lobeless embryos of *Dentalium vulgare,* we have repeated these experiments, as we needed reference-embryos for our experiments on development following removal of the D-quadrant at successive cleavage stages. The first (Fig. 6) and second polar lobes (Fig. 7) were removed and development was observed. Our results, which included 16–20 embryos in each case, were in agreement with those of Van Dongen and Geilenkirchen (1975) and Van Dongen (1976a, b). They confirmed the results of Wilson (1904), Verdonk et al. (1971), on *Dentalium dentale* and the results of Verdonk (1968a) on *Dentalium antillarum.* A summary of experiments with the ages of embryos at the time of fixation is presented in Table 1.

One-Half Embryos

AB 1/2-Embryos. The 27 AB 1/2-embryos studied were very uniform in character (Fig. 4). They tended to be radially symmetrical during cleavage and gastrulation although the prototroch often had gaps and was irregular in its placement around the vegetal pole. No apical tuft, or posttrochal region formed; neither did any posttrochal structures appear at later periods. Partial exogastrulae occurred in about 1/4 of the cases. All embryos developed short cilia on the

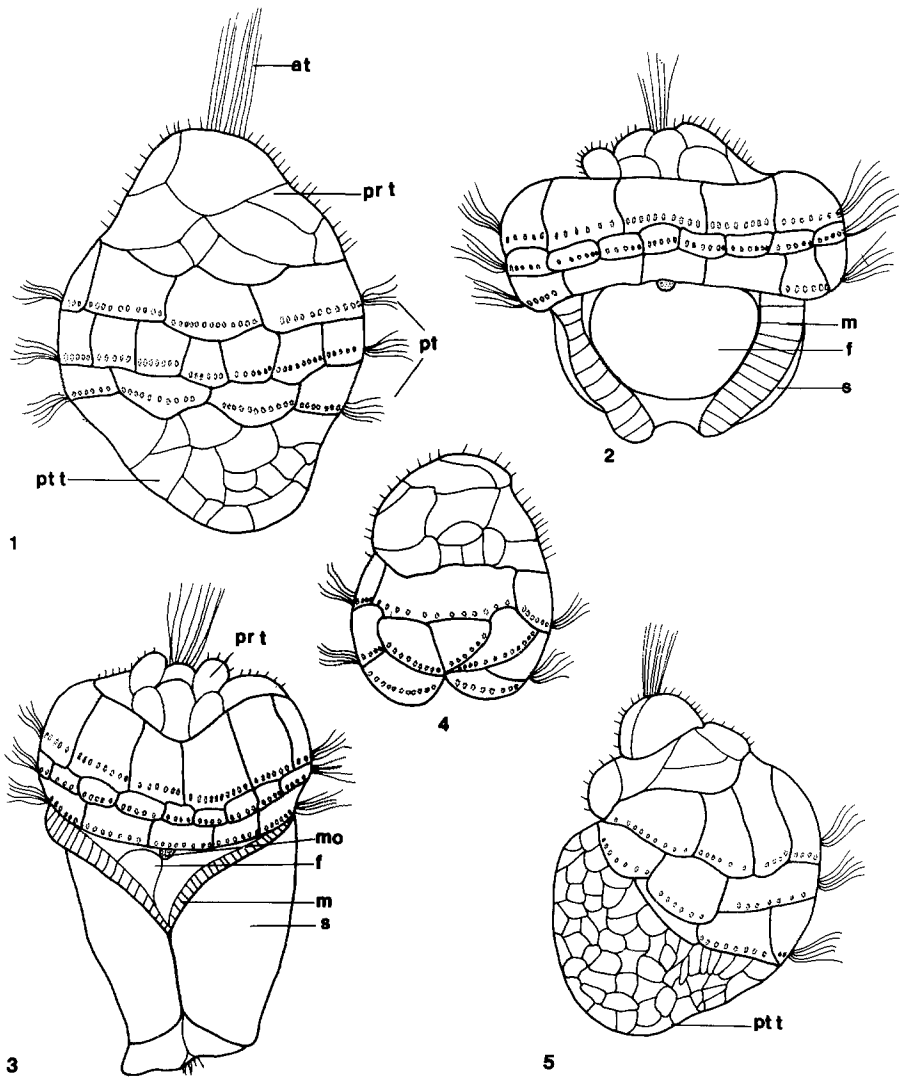

Fig. 1. Normal trochophore larva of *Dentalium* at about 28 h after fertilization. Apical tuft (*at*), pre-trochal region (*prt*), prototroch (*pt*), posttrochal region (*ptt*). The *small circles* in the prototrochal cells represent basal bodies. All drawings are camera lucida tracings of silver stained larvae × 230

Fig. 2. Control larva about 72 h after fertilization, undergoing major organogenesis. Foot (*f*), mantle (*m*), shell (*s*). × 230

Fig. 3. Control larva 4 days old. Elongation and gradual metamorphosis leads to settling about 2 days later. Foot (*f*), mantle (*m*), mouth (*mo*), pretrochal region (*prt*), shell (*s*). × 230

Fig. 4. AB 1/2 larva 48 h after fertilization. Note the absence of the apical tuft and posttrochal region and the poor organization of the prototroch. × 230

Fig. 5. CD 1/2 larva 48 h after fertilization. The apical tuft is asymmetrically located. The prototroch is abnormal and the posttrochal region (*ptt*) is unusually large. × 230

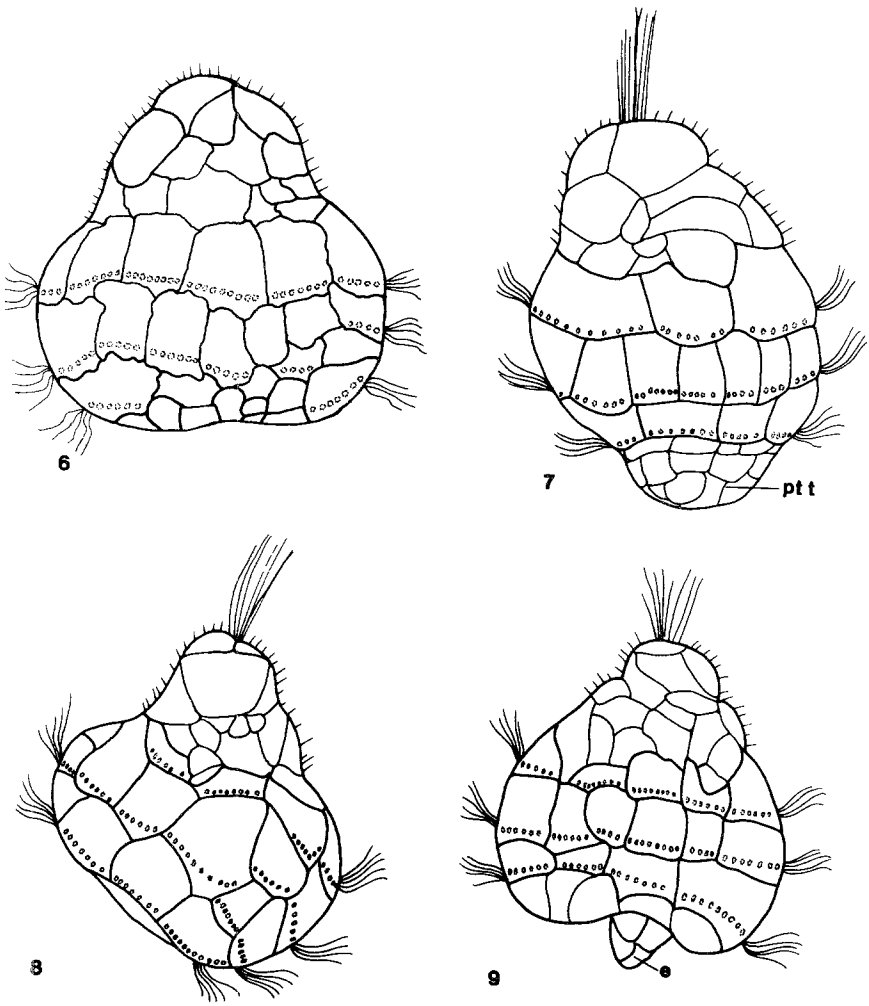

Fig. 6. Larva at 72 h after removal of the first polar lobe. The apical tuft and posttrochal region are missing. The lower prototroch is incomplete. × 230

Fig. 7. Larva at 72 h after removal of the second polar lobe. The apical tuft is present, the prototroch is complete and there is a reduced posttrochal region (*ptt*). × 230

Fig. 8. Larva at 48 h after removal of the D macromere. The apical tuft is present, the prototroch is asymmetrical and the posttrochal region is essentially absent. × 230

Fig. 9. Larva 72 h after removal of the 1D macromere. The apical tuft is present, the prototroch is uniform and there is a vegetal extrusion of endoderm (*e*). × 230

pretrochal cells. The one exogastrula sectioned failed to form a gut cavity while the 4 gastrulated embryos sectioned have a clearly defined enteron.

CD 1/2-Embryos. A total of 16 CD 1/2-embryos were prepared as whole mounts, and 5 were sectioned. Cleavage was partial; the time of cleavage was the same

as in control embryos, and the polar lobe of the second cleavage was typical in all respects. CD 1/2-larvae were never radially symmetrical but showed definite defects due to the absence of the AB quadrant blastomeres. The apical tuft is dislocated toward the side and the prototroch is incomplete on the same side (Fig. 5). The posttrochal region is relatively larger than in the control embryos but never forms normal posttrochal structures. No shell or shell gland was observed but extensions of the epithelium indicate an attempt at mantle formation. Statocysts were present in 3 of the 5 sectioned embryos, a gut cavity was usually present. No cerebral pits were observed in the pretrochal region. None of these embryos formed a recognizable foot but ciliated columnar epithelial patches typical of the foot were observed in the general region of the statocyst.

Deletion of D-Quadrant Macromeres

ABC-Embryos (−D). Embryos developed in a consistent and uniform way with little variation after removal of the D-macromere. Whole mounts were prepared of 34 larvae and 7 were sectioned. Cleavage was partial and recognizably tripartite even in the trochophore. Four of the 41 embryos carefully studied lacked apical tufts, but these structures were well developed in the rest (Fig. 8). Gastrulation was usually complete, although small endodermal protrusions at the vegetal pole were observed in 3 cases. The prototroch usually consisted of 3 rows and gaps were seldom present. The posttrochal region was absent in the trochophore and no posttrochal structures ever formed. These embryos resemble those from which the second polar lobe was removed. Endodermal differentiation is similar to that of lobeless embryos where the enteron remains anterior in position and differentiates into typical liver tissue. No hindgut can be distinguished.

ABC+1d-Embryos (−1D). These embryos which have all the cells of the 1st quartet present have better prototroch development than −D-larvae. The prototroch usually is of the typical evenly spaced three rows without gaps. Of the 37 embryos prepared as whole mounts and the 11 sectioned embryos, 12 were exogastrulae with the endodermal protrusions ranging from small (Fig. 9) to large. In living embryos, these endodermal protrusions appeared to resemble the posttrochal region but careful examination revealed that the resemblance was superficial. Neither the posttrochal region nor any posttrochal structures formed. The apical tuft was normally located in those larvae which gastrulated completely but was often toward one side in exogastrulae. The endoderm in exogastrulae formed no enteron, whereas those which completed gastrulation appear to have one.

ABC+1d+2d-Embryos (−2D). A total of 22 embryos were prepared as whole mounts and 6 were sectioned. In more than 2/3 of these embryos the pretrochal region appeared normal. The apical tuft is central and well formed, the three rows of the prototroch are symmetrical and unbroken and cephalic pits appear near the end of the second day, which is typical morphogenesis. Only two

embryos lacked the apical tuft and these also lacked most of the posttrochal ectoderm. The posttrochal ectoderm never completely covered the endoderm (Fig. 10); the endoderm in some cases protruded as a column which was partly covered by ectoderm. A single statocyst was present in 6 embryos, and in 12 embryos a small foot appeared to be formed. No shell gland or shell ever formed. The endoderm developed diverticulae but these did not show any additional differentiation.

ABC+1d+2d+3d-Embryos (−3D). Sixteen larvae of this type were prepared as whole mounts and 4 were sectioned. These embryos are indistinguishable from controls except for an early asymmetry which marks the site of the deleted cell. This condition has usually disappeared by the gastrula stage and is only rarely detectable afterwards. The trochophores (Fig. 11) have typical apical tufts, pretrochal regions, prototrochs and only slightly reduced posttrochal regions. Cerebral pits form, as do the typical posttrochal structures, the shell gland, the paired mantle folds and the foot with paired statocysts. The gut appears to be complete at least in some cases. The mesenchyme is so dense that no distinction could be made between control and operated embryos in this regard.

ABC+1d+2d+3d+4d-Embryos (−4D). A total of 5 whole mounts, and 4 sectioned embryos were prepared. The 4D macromere is relatively small in *Dentalium* and no defects of any kind can be observed an hour after the operation. There is no apparent asymmetry. All pretrochal and posttrochal organs are of normal size and shape and appear at the same time as in controls.

Deletion of Micromeres

ABC+1D-Embryos (−1d). Sixteen embryos were prepared as whole mounts. Van Dongen and Geilenkirchen (1974) have shown by the study of cell lineage that the apical tuft is formed from derivatives of 1c and 1d. Embryos with 1d removed had apical tufts but with fewer cilia than controls. In 6 embryos from which 1c was deleted, the number of apical cilia was reduced. In 3 cases both 1c and 1d were deleted from the same embryo and no apical tuft formed. All other aspects of development in these embryos appeared normal, except that there may be a reduction in the size of the cerebral pits. This cannot be adequately analysed in existing preparations.

ABC+1d+2D-Embryos (−2d). A total of 27 whole mounts and 7 sectioned embryos were prepared. These embryos show considerable morphological variability but are fairly consistent in structures formed. The apical tuft was present in all except two embryos. Cerebral pits were not observed. The prototroch had a small gap in 6 cases but was otherwise well formed. Foot tissue was found in 3 of the sectioned embryos and a statocyst in one. Areas of small celled posttrochal ectoderm present in 12 whole mounts probably represent foot epithelium. No shell gland nor shell materials were observed. Eighteen of these embryos failed to gastrulate completely, and of these 6 were exogastrulae. A stomodeum appears to be present in at least 8 embryos.

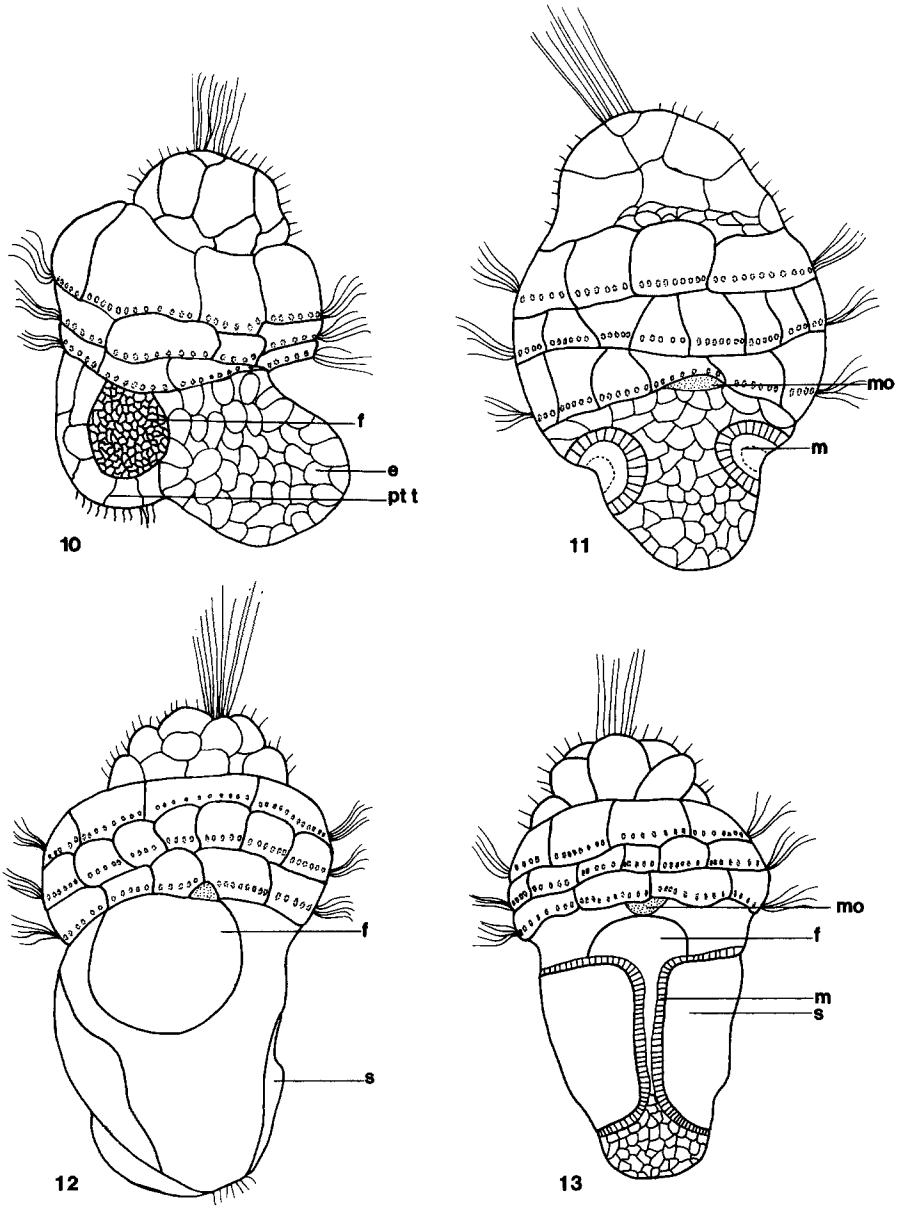

Fig. 10. Larva 24 h after deletion of the 2D macromere (ABC+1d+2d embryo). The apical tuft is present, the prototroch is essentially normal. There is some posttrochal ectoderm and the endoderm extrudes through it. Endoderm (*e*), Foot (*f*), posttrochal region (*ptt*). ×230

Fig. 11. Larva 46 h after deletion of the 3D macromere (ABC+1d+2d+3d). It is essentially indistinguishable from the controls. Mantle (*m*), mouth (*mo*). ×230

Fig. 12. Larva 72 h after deletion of the 4D macromere (ABC+1d+2d+3d+4d). The larva is externally indistinguishable from the controls although slightly younger in appearance. Foot (*f*), shell (*s*). ×230

Fig. 13. Larva 72 h after deletion of the 4d micromere. (ABC+1d+2d+3d+4D). It is indistinguishable from the controls. Foot (*f*), mantle (*m*), mouth (*mo*), shell (*s*). ×230

ABC+1d+2d+3D-Embryos (−3d). Nine whole mounts and 5 sectioned embryos of this type were prepared. The pretrochal region appeared normal in all cases with typical apical tufts and cerebral pits. The prototroch was normal. Shell appeared to be present and well formed except in two cases. The foot was present in all cases; in two cases it was greatly reduced and in the remainder somewhat reduced. Two embryos lacked statocysts, the rest had one statocyst except for one embryo which had two.

ABC+1d+2d+3d+4D-Embryos (−4d). Eight such embryos were prepared, one was sectioned. These appear perfectly normal (Fig. 13). They are so packed with mesenchyme that no defects of mesoderm are apparent.

Discussion

Since Wilson's (1904) classical work on the influence of the polar lobe on the development of *Dentalium,* eggs with polar lobes have been studied extensively to elucidate the influence of cytoplasmic localizations on development. Wilson showed that after removal of the lobe at first cleavage both the apical tuft and the posttrochal region were absent. Removal of the lobe at second cleavage had the same effect except for the apical tuft, which was present in most −pl 2 embryos. Wilson supposed that the factor essential for the formation of the apical tuft moved from the first lobe upward to the animal pole and became incorporated in the D-quadrant exclusively, since in isolation experiments of the micromeres at the 8-cell stage only the 1d micromere formed an apical tuft. Van Dongen and Geilenkirchen (1974) demonstrated that the apical tuft is located on the cells $1c^{1111}$ and $1d^{1111}$, which are derived from the 1c- and 1d-micromeres. In order to bring their data in accordance with Wilson's observation that after isolation of the micromeres only the 1d micromere formed an apical tuft and not the 1c micromere, they put forward the hypothesis that in normal development the cell $1c^{1111}$ can only form a part of the apical tuft when it received an inductive stimulus from the D-quadrant, most probably from the cell $1d^{1111}$. Since, however, in our experiments not only in CD-embryos the tuft is present but also in -D embryos, although reduced, the theory of Van Dongen and Geilenkirchen is untenable. Most probably Wilson's statement that only the 1d micromere can form an apical tuft is incorrect. The reason may be that he based his conclusion on too few cases of isolated first quartet cells. As removal of the second polar lobe or of the D blastomere at the 4-cell stage does not result in the absence of the apical tuft, determinants for apical tuft formation are not contained in the second lobe but are divided over the C and D blastomeres at second cleavage. This does not mean, however, that the determinants for the apical tuft, localized in the first polar lobe, move upward already at the 2-cell stage so that they do not become incorporated in the second lobe. Geilenkirchen et al. (1970) found that about 60% of the first polar lobe from the vegetal side can be removed without affecting the formation of an apical tuft. As they also established that the second lobe amounts to about 65% in volume of the first lobe, one may suppose

that determinants for apical tuft formation are localized in the apical part of the first polar lobe, which is not incorporated in the second lobe but segregated to both the C and D blastomeres at second cleavage.

The influence of the polar lobe on morphogenesis of the pretrochal region is not restricted, however, to the apical tuft. Van Dongen (1976b) showed that after removal of the polar lobe four cerebral ganglia are formed instead of the two found in the normal embryo. Although one might expect cerebral pits or ganglia to be formed in the CD, −D or −1D embryos, paired cerebral pits were observed only in the −2D, −3D and −4D embryos. This problem needs further investigation. The −pl 2 and the −D embryos do not form a posttrochal region. The question therefore arises, as to how such determinants are segregated during further cleavage of this quadrant. From our data it is clear that −1D (ABC+1d) also develops in the same way as a −pl 2 embryo. Consequently at third cleavage the lobe material is restricted to the 1D blastomere.

The second quartet of micromeres includes the first somatoblast 2d, a cell of great importance for morphogenesis, as it forms the shell gland and part of the foot (Van Dongen and Geilenkirchen 1974). In accordance with these data the −2d (ABC+1d+2D) showed a partial development of the posttrochal region, including a small foot with one statocyst, but shell gland or shell were never observed. This may be due to partial exogastrulation which occurred in most of the −2d embryos. As, however, foot tissue including statocyst was formed, an inductive influence from 2D or 3D on shell formation cannot be excluded.

The third quartet includes the 3d blastomere, from which a part of the foot is derived (Van Dongen and Geilenkirchen 1974). Indeed −3d embryos show a reduction of the foot and −3D embryos have a normal foot and shell. Eggs in which the macromere 4D is deleted, develop into normal larvae, which apparently do not lack any structures. Consequently in *Dentalium* as in *Ilyanassa* (Clement 1962) the 4D macromere is nutritional in nature. In molluscan embryology 4d is called the mesentoblast, as descendants of this cell produce besides some entoderm the adult mesoderm. After removal of 4d no defects in the larvae could be detected. This does not mean that the 4d is of no importance for the development of *Dentalium*. As the larval mesoderm, derived from $2a^{212}$ and $2c^{122}$ is well developed in *Dentalium* (Van Dongen and Geilenkirchen 1974) the extensive larval mesenchyme in the −4d embryos may obscure the absence of adult mesoderm. Also there is much less regular organization of the mesenchyme in the operated embryos than in the normal embryos investigated by Van Dongen (1977).

Our data are in general agreement with the data of Clement (1962) who removed the D macromere at successive stages in the gastropod *Ilyanassa*. Also in this snail lobeless, ABC, ABC+1d and ABC+1d+2d develop into partial larvae, which fail to form most of the adult structures. In both molluscs the shell is missing after deletion of 2D, although the 2d cell is present, which gives rise to the whole shell in *Dentalium* (Van Dongen and Geilenkirchen 1974) and to most of the shell in *Ilyanassa* (Cather 1967). Raven (1952) has put forward the theory that in *Lymnaea* the shell gland is induced by the

small-celled endoderm. Later Hess (1962, 1971) supposed that this influence was not restricted to this small-celled endoderm but that the large-celled endoderm is capable of inductive interaction as well. Cather (1967) showed that the mesentoblast 4d and even an isolated polar lobe could induce the formation of an external shell by an isolated ectoblast. As, however, removal of 2d in *Dentalium* and of 2c+2d in *Ilyanassa* results in the absence of a shell, the normal competence for shell formation is restricted to special cells.

The inductive influence for shell formation is also limited to the D-quadrant and as −3D embryos both in *Dentalium* and *Ilyanassa* form an external shell, the inductive influence has been exerted prior to removal of the 3D. Clement (1962) got some indication that a fuller differentiation of the shell was obtained, when 3D was removed at a late stage in the interval between the fifth and sixth cleavage, which he attributed to an inductive influence of 3D. Also in our experiments 3D was removed late in this interval between the fifth and sixth cleavage and this cell may have influenced the morphogenesis in the 2d-cell-line, which without this support cannot form a shell. Van Dongen (1976a, b) by comparing the cell-lineage of normal and lobeless embryos of *Dentalium* got strong indications that the D-quadrant influences morphogenesis in the A- and C-quadrant. For this influence the segregation of the material of the polar lobe into the D-quadrant is essential. Within this quadrant the polar lobe material is segregated according to a fixed pattern, by which cells are specified and obtain a developmental capacity in distinct directions. For full determination of the D-quadrant cellular interactions may nevertheless be indispensable.

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