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Title	Biology of Dicyemid Mesozoans - Taxonomy, Morphology, and Embryology
Author(s)	古屋, 秀隆
Citation	大阪大学, 1994, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3075002
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博士論文

Biology of Dicyemid Mesozoans-

Taxonomy, Morphology, and Embryology

中生動物ニハイチュウの生物学-
分類・形態・発生

平成6年2月

大阪大学大学院理学研究科生理学専攻

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Doctoral Dissertation

Biology of Dicyemid Mesozoans- Taxonomy, Morphology, and Embryology

By

Hidetaka Furuya

February, 1994

CONTENTS

INTRODUCTION

Very small and simple parasitic organisms now called dicyemids were discovered in 1839 by Krohn from the renal sac of benthic cephalopod molluscs [10]. In 1876, Edward van Beneden made the first systematic study of the dicyemids using histological methods, and felt that he had evidence for a true "missing link". Here was a group of animals that seemed to bridge the gap between the Protozoa and the Metazoa. Thus, he applied the dicyemids the name "Mesozoa" as intermediate between the Protozoa and the Metazoa [1]. Since van Beneden's pioneering study, they have been studied by a number of zoologists, including Whitman [25], Hartmann [3], Lameere [11, 12], Nouvel [19, 20], Gersch [2], Hyman [7, 8], Stunkard [24], McConnaughey [16, 17, 18], and others. There are almost as many different interpretations of their morphology, life cycle, and phylogeny as there are zoologists that have studied them. Lameere asserted that the Mesozoa was degenerate Echiuroidea. Whitman, Nouvel, and Stunkard considered that the Mesozoa was the flatworm (Platyhelminthes), secondarily simplified as a result of parasitism. McConnaughey had an idea that they represented an offshoot from a primitive stock of planuloid, acoelous, ciliated worms related to very early progenitors of the flatworms or certain of the earliest aschelminths. Hyman believed that their characters were in the main primitive and not the result of parasitic degeneration. Recent studies on the base compositions and sequences of their nucleic acids have suggested that dicyemids are somewhat closer to ciliate protozoans than to

flatworms [14, 22]. Thus, it is still debated whether dicyemids are truly primitive multicellular animals [1, 7, 8, 13, 14, 15, 22] or they are actually organisms that have degenerated as a result of parasitism [3, 12, 20, 24, 25].

The bodies of dicyemids consist of only 20 to 40 cells and they are organized very simply. Two kinds of adult forms, nematogens and rhombogens, are found in dicyemids. Both are composed of a limited number of outer ciliated cells and a single elongated internal cell (axial cell). Asexual reproduction occurs within the axial cell of nematogens and a vermiform embryo develops from an agamete (axoblast). Sexual reproduction takes place within the axial cell of rhombogens. A hermaphroditic gonad, which is called an infusorigen, is formed within the axial cell of rhombogens and fertilization occurs around the infusorigen. The zygote undergoes cleavages and develops into an infusoriform larva within the axial cell. The transition from the nematogen phase to the rhombogen phase may be correlated with increasing population density [13, 15]. The infusoriform larvae are shed into the sea with the urine of the host, but the process of entry of the infusoriform larva into the cephalopod body and further development from the infusoriform larva to the vermiform stage are unknown. A stem nematogen is believed to develop asexually from a germinal cell of the infusoriform larva [15, 17, 20, 21, 24], but such a developmental process is not confirmed. No intermediate host may intervene between two cephalopod hosts [5, 13, 15].

Systematic knowledge of the dicyemids is embodied in several works dealing primarily with species in Europe and North America.

Those in Japan are largely unknown; the only published accounts of them are two records of dicyemid species by Nouvel and Nakao in 1938 [19] and by Nouvel in 1947 [20]. Since then, no reports on the dicyemids from Japan have been published. In the last five years, I have examined the dicyemids in the renal sacs of seven species of japanese cephalopods, namely, Octopus vulgaris, Octopus minor, Octopus fangsiao, Octopus dofleini, Sepioteuthis lessoniana, Sepia esculenta, and Sepia lycidas. Nineteen dicyemid species, including four described species, were observed in their renal sacs. I could characterize at least two species that are different from the other species so far described. Chapter I deals with description of these two new dicyemids species; one from Octopus vulgaris and the other from Octopus minor. A detailed description of the infusoriform embryos of Dicyema misakiense Nouvel et Nakao and an additional account of Dicyema acuticephalum Nouvel are also given.

In any attempt to evaluate the phylogenetic position of an organism, a knowledge of the development of the organism can be crucial. In the case of dicyemids, such information may also attract the attention of developmental biologists because, in the animal kingdom, the development of dicyemids may represent one of the simplest patterns of cell differentiation that occurs during embryogenesis. A complete cell lineage of dicyemids may provide a basis to understand the process of cell differentiation in multicellular animals. Nevertheless, the development of the dicyemid has been minimally studied [14, 17, 21]. Chapter II deals with the development of infusoriform embryos; the pattern of cleavage and the cell lineage during the development of the

infusoriform embryo of Dicyema japonicum is described.

Dicyemids have very simple hermaphroditic gonads (infusorigens) that are composed of a very small number of cells. Infusorigens may also prove to be useful as a model system for studies of differentiation of gametes, such as the gonad of the nematode Caenorhabditis elegans [4, 9]. However, the development of infusorigens has been studied only sporadically [12, 17, 20]. Chapter III deals with the development of the infusorigens; the patterns of the development of the infusorigens in four species, D. orientale, D. acuticephalum, D. japonicum, and D. misakiense are described systematically. In addition, the fecundity of infusorigens in four species is discussed.

Vermiform embryos that develop asexually from agametes are much simpler in body organization than infusoriform embryos that develop from zygotes. The development of vermiform embryos was described in the early literatures [2, 10, 18, 20], but the pattern of cell divisions and the process of cell arrangement during embryogenesis remain to be established. Chapter IV deals with the development of the vermiform embryos; the pattern of division and cell lineage during the development of the vermiforms of D. acuticephalum and D. japonicum is described.

The life cycle of the dicyemids is not elucidated in one important aspect, that is the develpoment of infusoriform larvae. The minute size of the infusoriforms renders any attempt to follow them in the ocean practically hopeless. If in vitro culture system is established in the laboratory, new information on dicyemid life cycle would be obtained. Although some attempt to culture the dicyemids in vitro has been made [14, 23], no

crucial information about their life cycle is available. In addition, the embryos of dicyemids are consistently composed of a very small number of cells. These cells, with more or less clear evidence of specific differentiation, are produced after only a very few rounds of cell division. Thus, these embryos might be useful as the simple model system for the study of cell differentiation and morphogenesis in animals, especially when in vitro culture of these embryos becomes available. In the studies described in Chapter V, the survival times of nematogens and infusoriform larvae in the octopus urine and in seawater were determined. An attempt to culture the dicyemids in an artificial medium was also made.

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CHAPTER I

Two New Species of the Genus Dicyema (Mesozoa) from Octopuses of Japan with Notes on D. misakiense and D. acuticephalum*

* Note: Chapter I is a part of the doctoral dissertation and is not an official description of new species. The official description of the new species was given elsewhere (Furuya et al., Zool. Sci., 9, 423-437, 1992)

INTRODUCTION

The first record of dicyemid mesozoans in Japan was published in 1938 by Nouvel and Nakao [1]. They found Dicyema misakiense NOUVEL et NAKAO, 1938, in the renal sac of Octopus vulgaris, and also Dicyema orientale NOUVEL et NAKAO, 1938, in Sepioteuthis lessoniana. Later, Nouvel [2] described Dicyema acuticephalum NOUVEL, 1947, which was also obtained from Octopus vulgaris, and identified another dicyemid from Sepia esculenta as Pseudicyema truncatum WHITMAN, 1883, which had been already described in Europe. All these host cephalopods were collected in the waters close to the Misaki Marine Biological Station of the University of Tokyo. Since then no reports on the dicyemid species from Japan have been published.

We examined the dicyemids in the renal sacs of octopuses caught off the coast of Japan, and we found at least two new dicyemids that are distinctly different from each of the four species mentioned above and from the other species so far described in various regions outside Japan.

The present paper deals with these two new dicyemid species: one obtained from Octopus vulgaris and the other from Octopus minor. In addition, we give a detailed description of the infusoriform embryos of Dicyema misakiense NOUVEL et NAKAO and further give an account of Dicyema acuticephalum NOUVEL.

MATERIALS AND METHODS

From April of 1989 to May of 1991, 21 individuals of Octopus vulgaris and 19 of Octopus minor were obtained for detection of dicyemids. Most of them were obtained from fish markets and fishermen, but two were caught by the authors. The size, sex, and source of each of these octopuses are given in Tables 1 and 2.

From every octopus, which was brought alive to the laboratory, the head and tentacles were cut off just behind the eyes, without anesthetic. Then the visceral hump was placed, ventral side up, in a tray, and the mantle was opened to expose the paired renal sacs. Pieces of renal tissues were smeared with the fluid from the renal sac on slide glasses. Some preparations were used to confirm the occurrence of living dicyemids under the phase-contrast microscope, while others were promptly fixed in Carnoy's fluid or in alcoholic Bouin's fluid (a 15:5:1 mixture of saturated picric acid in absolute ethanol, formalin, and acetic acid). Carnoy-fixed preparations were subjected to Feulgen or McMannus's periodic-acid Schiff (PAS) procedures, and then they were stained with Ehrlich's acid hematoxylin and light green. Alcoholic Bouin-fixed preparations were subjected to the last two staining procedures only. After the staining, the preparations were dehydrated and mounted in the usual fashion for observation of dicyemids under the light microscope.

Measurements and drawings were made with the aid of a micrometer and an image tracer, respectively.

Dicyemidae VAN BENEDEN, 1882

Dicyema KOLLIKER, 1849

Dicyema japonicum sp. nov. FURUYA et TSUNEKI

[New Japanese name: Yamato-nihaityu]

(Figs. 1-7, Tables 1, 3, and 4)

Host: Octopus vulgaris Lamarck, Octopodidae.

Locality: Western Honshu, Japan. See "Source" in Table 1.

Syntypes: A slide registered as NSMT-Me-1 was deposited at the Institute of Natural History, National Science Museum of Tokyo, Tokyo, Japan. This slide was prepared from No. VU44 octopus (Table 1) and contains both nematogens and rhombogens. It includes D. misakiense as well, but D. japonicum can be clearly distinguished from D. misakiense in the shape of the head as described below. Other slides were numbered according to the host number in Table 1 and are in the authors' collection.

Etymology: The specific name "japonicum" was given, because this species is common in Octopus vulgaris caught off the coast of Japan.

DESCRIPTION

Diagnosis: Body length up to 1800 μm. Peripheral cell number of vermiform phases (vermiform embryo, nematogen, and rhombogen) usually 22: 4 propolars, 4 metapolars, 14 trunk cells. Propolar

and metapolar cells form a disc-like head together with parapolar cells. Infusoriform embryos consist of 37 cells. Nucleus number of urn cell, 1. Host, Octopus vulgaris.

Nematogens (Figs. 1-3): Body slender, 300 to 1800 μm long; 40-75 μm wide. Peripheral cell number usually 22 (Table 3): 4 propolars, 4 metapolars, 2 parapolars, 10 diapolars, 2 uropolars. Calotte and parapolar cells form cephalic enlargement. Calotte becomes disc-shaped as individuals grow. Cilia covering calotte about 4.7 μm long, oriented forward, slightly shorter but denser than cilia of trunk cells. Cytoplasm of both propolar and metapolar cells, stained by hematoxylin more conspicuously than that of other cells; PAS-positive, but negative after saliva test. Cells and nuclei of propolars, smaller, respectively, than those of metapolars. Trunk mostly uniform in width. Trunk cells, arranged in opposed pairs. No verruciform cells. Axial cell, cylindrical and rounded anteriorly, extends forward to base of propolar cells. Usually two sizes of axoblasts, standard and large; the latter often twice the size of the former. In large individuals, about 80 vermiform embryos at most found in the axial cell, calotte occasionally flower-like in shape, and a few accessory nuclei evident in both peripheral and axial cell. In Carnoy-fixed large individuals, many fine granules found in peripheral cells.

Transitional individuals from nematogens to rhombogens enclose degenerating vermiform embryos, proliferating infusorigens, and developing or full-grown infusoriform embryos, simultaneously, in the axial cell. Standard axoblasts only, no large ones in these individuals.

Vermiform embryos (Fig. 4): Full-grown vermiform embryos, 40 to 70 μm long, 8 to 12 μm wide; peripheral cell number fixed at constantly 22 (Table 3). The ratio of total body length to calotte length, 1:0.24 to 0.28. Anterior end of calotte tapering slightly and pointed bluntly. Trunk cells, arranged in opposed pairs. Axial cell tapering anteriorly, sometimes pointed, extending forward to base of propolar cells, as seen in nematogens. Axial cell nucleus, usually located in center or in anterior half of axial cell, always anterior to one or two standard-sized axoblasts.

Stem nematogens (Fig. 5): Two stem nematogens were obtained. One of them, 221 μm long, with three axial cells. Peripheral cell number: 23 (4 propolars, 4 metapolars, 2 parapolars, 11 diapolars, 2 uropolars). Vermiform embryos found in both second and third axial cell but not in the first. The other stem nematogen, 223 μm long, also with three axial cells. Peripheral cell number: 26 (4 propolars, 4 metapolars, 3 parapolars, 14 diapolars, 1 uropolars). Vermiform embryos found in all three axial cells. Only standard-sized axoblasts in both stem nematogens.

Rhombogens (Figs. 1-3): Slightly shorter and stockier than nematogens, otherwise generally similar in shape and proportion; body 300 to 1000 μm long; 40 to 75 μm wide. Peripheral cell number, usually 22, sometimes lower (Table 3). Cephalic enlargement, composed of calotte and parapolar cells as in nematogens. Calotte becomes disc-shaped as individuals grow. Shape and tip position of axial cell, similar to those in nematogens. Axial cell sometimes expanded at the region where

infusorigens are included. One, sometimes two, and very rarely three infusorigens in the axial cell. Some accessory nuclei observed occasionally in both peripheral and axial cell. No verruciform cells.

Infusorigens (Fig. 6): Medium-sized, sometimes relatively large. Axial cell usually irregular in shape. In 25 mature infusorigens examined: number of external cells including oocytes, 8 to 58 (mode, 15); number of internal cells including spermatocytes, 3 to 19 (mode, 3). Fertilized eggs, 12.3 μm in diameter.

Infusoriform embryos (Fig. 7): Ovoid, rounded bluntly to pointed posteriorly. Based on 100 full-grown embryos, length (excluding cilia), 23.68 ± 1.98 μm (mean ± S.D.); lenth-width-height ratio, 1:0.80:0.73. Cilia at the posterior end, 6 to 7 μm long. Refringent bodies, smaller than total mass of four urn cells, occupy anterior one-third or so of embryo, when viewed from lateral side. Nuclei of second ventral cells, small and pycnotic. Ventral internal cells project cilia to urn cavity. Capsule cells with many large granules on side adjacent to urn. Granules, intensely stained by PAS procedure and staining-resistant in saliva test. Full-grown infusoriform embryos consist of 37 cells: 33 somatic and 4 germinal cells (Table 4). Cell terminology used here is that of NOUVEL [3] and SHORT and DAMIAN [4]. Somatic cells are composed of peripheral cells that cover a large part of anterior and lateral surfaces of embryo (2 enveloping cells), peripheral cells with cilia on external surfaces (2 paired dorsal cells, 1 median dorsal cell, 2 dorsal caudal cells, 2 lateral caudal cells, 1 ventral caudal cell, 2

lateral cells, 2 posteroventral lateral cells), peripheral cells with refringent bodies (2 apical cells --- cilia not clearly visible on these cells), peripheral cells without cilia (2 first ventral cells, 2 second ventral cells, 2 anterior lateral cells, 1 couvercle cell), internal cells with cilia (2 ventral internal cells), internal cells without cilia (2 dorsal internal cells, 2 capsule cells, 4 urn cells). Each of the four urn cells encloses its own nucleus and one germinal cell with its one nucleus (Table 4). All somatic nuclei tend to become pycnotic as infusoriform embryos mature.

Geographical variations: Not found either in vermiform stages or infusoriforms.

Dicyema misakiense NOUVEL et NAKAO, 1938

[Japanese name: Misaki-nihaityu]

(Fig. 8, Tables 4 and 5)

Materials examined: Slides were numbered according to the host number in Table 1 and are in the authors' collection.

Diagnosis: Body length up to 1700 μm. Peripheral cell number 22. Calotte consists of two tiers; the tier of propolars slightly smaller than that of metapolars and faintly constricted from the tier of metapolars. Infusoriform embryos, 37 cells. Nucleus number of urn cell, 1. Host, Octopus vulgaris.

Note: Nouvel and Nakao [1] reported that the number of peripheral cells in vermiform phases was usually 22, but was

sometimes lower. However, we found this number to be almost constant; 157 out of 158 vermiform individuals examined had 22 peripheral cells, but only one nematogen had 21 cells (Table 5).

The original description of the infusoriform embryos is brief: the embryos (excluding cilia) are 25 μm long, 20 μm wide, and 18 μm high; and each of the urn cells has its own nucleus and one germinal cell [1]. We confirmed in our materials that the nucleus number of urn cells is one (Table 4). Additional details of the infusoriform embryos are given, based on our observations.

Infusoriform embryos: Ovoid and rounded bluntly to pointed posteriorly. Based on 100 full-grown embryos, length (excluding cilia), 24.67 ± 1.28 μm (mean ± S.D.); length-width-height ratio, 1:0.86:0.81. Cilia at the posterior end, 6 to 7 μm long. Refringent bodies, smaller than total mass of four urn cells, occupy about 40% of anterior part of embryo, when viewed from lateral side. Nuclei of second ventral cells, small and pycnotic. Ventral internal cells with cilia projecting into urn cavity. Capsule cells contain many large granules, which are located on side adjacent to urn. Full-grown infusoriform embryos composed of 37 cells (33 somatic and 4 germinal). Somatic cell composition same as in D. japonicum (Table 4). Somatic nuclei tend to show pycnosis as embryos mature.

Dicyema acuticephalum NOUVEL, 1947

[New Japanese name: Togari-nihaityu]

(Tables 4 and 6)

Materials examined: Slides were numbered according to the host number in Table 1 and are in the authors' collection.

Diagnosis: Body length up to 800 μm. Peripheral cell number constantly 18. Calotte conical in shape. Parapolars bulging behind the calotte. Infusoriform embryos consisting of 37 cells. Nucleus number of urn cell, 2. Host, Octopus vulgaris.

Note: Nouvel [2] reported that the number of peripheral cells in this species ranged from 16 to 19 (generally 18), and the nucleus number of urn cells was two. We confirmed his findings in our materials, but the number of peripheral cells in the vermiform embryos was constant and equal to 18 and no variation was detected (Tables 4 and 6). We also revealed that fully mature infusoriform embryos consist of 37 cells.

REMARKS

The distinction between Dicyema japonicum and Dicyema acuticephalum is clear because of differences in typical number of peripheral cells in full-grown vermiform phases (22 vs. 18) as shown in Tables 3 and 6, in calotte shape (disc type vs. conical type), in position of the axial cell tip (extending to the base of propolar cells vs. metapolar cells), and in the number of

somatic nuclei in the urn cells of infusoriform embryos (one vs. two) as shown in Table 4.

Both Dicyema japonicum and Dicyema misakiense are similar in the average length of the body and have, typically, 22 peripheral cells (Tables 3 and 5). In both species, the axial cell extends to the base of propolar cells in vermiform phases, and infusoriform embryos consist of 37 cells, which include urn cells that contain their own single nuclei (Table 4). Nevertheless, we can point out the following differences between D. japonicum and D. misakiense. (1) The calotte of D. japonicum is a disc-shaped and forms the cephalic enlargement with parapolar cells (Figs. 2 and 3), whereas D. misakiense has a slender calotte (Fig. 8). No individuals with a calotte that was intermediate in shape between those of the two species were found. (2) Vermiform embryos of D. japonicum often have two axoblasts within the axial cell, while those of D. misakiense consistently have only one axoblast [1]. (3) The length-width-height ratio of infusoriform embryos of D. japonicum is different from that of D. misakiense (1:0.80:0.73 vs. 1:0.86:0.81). These differences should be sufficient for establishing species in the dicyemids and therefore we have identified D. japonicum as a new species. In the paper by Nouvel and Nakao [1], the text in lines 33-36 on p. 74, in lines 1-5 on p. 75, and Figures 6 and 7 on p. 76 could be regarded as referring to D. japonicum and not to D. misakiense.

Dicyema aegira McCONNAUGHEY and KRITZLER, 1952 [5], from Octopus vulgaris, has 22 peripheral cells as equal as D. japonicum, but the two species are easily distinguishable from

the following differences. D. aegira has a calotte that is slightly longer than the breadth of the anterior end of the trunk, and the axial cell ends at the base of the metapolar cells, unlike the arrangement in D. japonicum.

Dicyema orientale NOUVEL et NAKAO, 1938 [1] has a calotte that is very similar in shape to that of D. japonicum, and it also has 22 peripheral cells. However, at full-grown vermiform stages, D. orientale is much larger than D. japonicum; the number of axoblasts within vermiform embryos in D. orientale is also larger than in D. japonicum (8 vs. 1 or 2). The host cephalopod of D. orientale is Sepioteuthis lessoniana, a decapod, not an octopod. It seems reasonable, therefore, that these two dicyemids should be considered members of different species.

Dicyema benthoctopi HOCHBERG et SHORT, 1970 [6] is very similar to D. japonicum in the calotte shape and in the size of vermiform stages, but the number of peripheral cells in D. benthoctopi is very variable. The host of D. benthoctopi is Benthoctopus magellanicus, a deep-sea octopod distributed in the Atlantic Ocean near the Falkland Islands, at about 500 km east of the Strait of Magellan, the southern extremity of the South American Continent. It is hard to conclude, therefore, that D. benthoctopi and D. japonicum are the same species, even though infusoriforms of D. benthoctopi have not yet been described.

Dicyemidae VAN BENEDEN, 1882

Dicyema KÖLLIKER, 1849

Dicyema clavatum sp. nov. FURUYA et KOSHIDA

[New Japanese name: Konbou-nihaityu]

(Figs. 9-14, Tables 2, 4, and 8)

Host: Octopus minor (Sasaki), Octopodidae.

Locality: Western Honshu, Japan. See "Sorce" in Table 2.

Syntypes: A slide registered as NSMT-Me-2 was deposited at the Institute of Natural History, National Science Museum of Tokyo, Tokyo, Japan. This slide was prepared from No. MI95 octopus (Table 2) and contains nematogens and rhombogens of D. clavatum exclusively. Other slides were numbered according to the host number in Table 2 and are in the authors' collection.

Etymology: The specific name "clavatum" is given, because of the shape of the body is characteristically clavate.

DESCRIPTION

Diagnosis: Body length up to 1000 μm. Peripheral cell number of vermiform phases usually 22: 4 propolars, 4 metapolars, 14 trunk cells. Propolar and metapolar cells flat and covering the enlarged end of the axial cell. The infusoriform embryos consist of 39 cells. Nucleus number of urn cell, 2. Host, Octopus minor.

Nematogens (Figs. 9-11): Body, pestle-like; 200 to 1000 μm long; 60 to 120 μm wide. Peripheral cell number usually 22; 4

propolars, 4 metapolars, 2 parapolars, 10 diapolars, 2 uropolars, diapolars sometimes lower (Table 8). Cephalic swelling distinct. Calotte, smoothly rounded and cap-shaped in most individuals, flattened and disc-shaped in a few individuals. Cilia covering calotte about 6 um long, oriented forward, more densely distributed than those on trunk cells. Trunk cells arranged in opposed pairs. Uropolar cells occasionally verruciform; their nuclei, often enlarged; their cytoplasm, stained intensely by hematoxylin. Axial cell extends anteriorly to base of propolar cells, typically enlarged and rounded in calotte region. Rounded tip of axial cell is covered by a cap of thin propolar cells and by metapolar cells often arranged as a band. Large individuals enclose about 80 vermiform embryos, and peripheral cells often decrease to 21, sometimes to 20, in number.

Vermiform embryos (Fig. 12): Full-grown vermiform embryos, 70 to 100 µm long; 12 to 18 µm wide; peripheral cell number fixed at 22 (Table 8). Ratio of total body length to calotte length, 1:0.17 to 1:0.19. Anterior end of calotte, rounded. Trunk cells, arranged in opposed pairs. Axial cell nucleus, usually located in the center of the cell, one or two axoblasts anteriorly.

Rhombogens (Figs. 9, 10 and 11): Slightly smaller than nematogens, 200 to 600 µm long; 60 to 140 µm wide. Shape of calotte similar to that in nematogens, although cephalic swelling more massive and broader as individuals grow. Cilia covering calotte, similar in length and in orientation to those of nematogens. Two uropolar cells, sometimes one additional cell

adjacent to uropolars, occasionally verruciform; these cells often with large nuclei, with cytoplasm staining intensely with hematoxylin. Shape of axial cell and arrangment of both propolar and metapolar cells with respect to axial cell, similar to nematogens.

Infusorigens (Fig. 13): Relatively small. Axial cell usually ovoid, about 12 to 17 μm in long axis. About one-third of axial cell surface, always exposed. In 25 infusorigens examined: number of external cells including oocytes, 5 to 12 (mode, 6); number of internal cells including spermatocytes, 2 to 5 (mode, 3). Fertilized eggs, about 12.1 μm in diameter.

Infusoriform embryos (Fig. 14): Ovoid and rounded bluntly to pointed posteriorly. Based on 100 full-grown embryos, length (excluding cilia), 24.10 ± 1.35 μm (mean ± S.D.); length-width-height ratio, 1:0.86:0.80. Cilia at posterior end about 7 to 8 μm long. Refringent bodies, about equal in size to total mass of four urn cells, occupy about 40% of anterior part of embryo, when viewed from lateral side. Nuclei of anterior lateral cells, small and pycnotic. Ventral internal cells project cilia into urn cavity. Short cilia growing from apical cells through dorsal fenestrae of enveloping cells. Full-grown infusoriform embryos consist of 39 cells (35 somatic and 4 germinal) and 43 nuclei in total (Table 4). Somatic cells composed of peripheral cells that cover anterior and lateral surfaces of embryo (2 enveloping cells), peripheral cells with cilia (2 apical cells, 2 paired dorsal cells, 1 median dorsal cell, 2 dorsal caudal cells, 2 lateral caudal cells, 1 ventral caudal cell, 2 lateral cells, 2 posteroventral lateral cells), peripheral cells without cilia (2

first ventral cells, 2 second ventral cells, 2 third ventral cells, 2 anterior lateral cells, 1 couvercle cell), internal cells with cilia (2 ventral internal cells), and internal cells without cilia (2 capsule cells, 2 dorsal internal cells, 4 urn cells). Each of four urn cells has two nuclei of its own and one germinal cell with its own nucleus (Table 4).

REMARKS

D. clavatum is very similar to D. robsonellae SHORT, 1971 [7], which was isolated from Robsonella australis in New Zealand, in the enlarged head and cap-shaped calotte. However, differences between D. clavatum and D. robsonellae are distinct in terms of the peripheral cell number in vermiform phases (22 vs. 20), in the cell number of infusoriform embryos (39 vs. 37), and in the occurrence of cilia on ventral internal cells (present vs. absent), respectively.

D. benthoctopi [6], D. orientale [1], and D. japonicum have 22 peripheral cells and an enlarged head, but each of these species has a conspicuously swollen and disc-shaped calotte, instead of a cap-shaped one. The hosts of these three species and D. clavatum are also different from one another.

O. minor is now regarded as a member of the O. macropus species complex [8]. D. paradoxum von KÖLLIKER, 1849, [2, 9] was isolated from O. macropus in Europe, but this dicyemid species could be easily distinguished from D. clavatum by the number of peripheral cells (25-28 vs. 22). D. clavatum is the

first dicyemid species obtained from O. minor to be described.

DICYEMID FAUNA IN Octopus vulgaris AND Octopus minor

In 20 out of 21 individuals of O. vulgaris, we found a total of four kinds of dicyemid, i.e., D. misakiense, D. acuticephalum, D. japonicum, and one more dicyemid not yet identified to species (Table 1). This unidentified dicyemid certainly belongs to the genus Dicyema because its calotte consists of 4 propolar and 4 metapolar cells that are not twisted in their arrangements; therefore this dicyemid is tentatively termed Dicyema sp.(A). As shown in Table 1, three species of dicyemids, including Dicyema sp.(A), were detected together in two hosts; two species of dicyemids, D. misakiense and D. japonicum in all cases, were found in 16 hosts; and a single species of dicyemid was found in four hosts. Only in one individual, listed as Host no. VU15, the smallest in size among the octopuses examined, no dicyemids at all were detected. In another small octopus, Host no. VU101, dicyemids were scarce, although two species were found in one of the renal sacs, while none were found in the other.

In Table 7 we have listed all the dicyemid species from Octopus vulgaris that we were able to find in the literature. It is apparent that a considerable number of dicyemid species have been recorded in association with this cephalopod.

In Octopus minor, nine out of nineteen individuals were infected by D. clavatum (Table 2). In five out of these nine individuals, in addition to D. clavatum, another species was also

found. This species clearly belongs to the genus Dicyema because of the cell composition and arrangement of the calotte, but the species is unidentified as yet and is named tentatively Dicyema sp. (B) (Table 2). In the octopus listed as Host no. MI92, this unidentified species was found in one renal sac, but D. clavatum was found in other one. Yet another species belonging to the genus Dicyema, tentatively named Dicyema sp. (C), was found in O. minor (Table 2), but it too has not yet been fully characterized.

SUMMARY

We examined dicyemid mesozoans from the renal sacs of both Octopus vulgaris and Octopus minor, obtained off the coast of Japan, and found two new species that belong to the genus Dicyema.

Dicyema japonicum sp. nov. from O. vulgaris, is a medium sized dicyemid, rarely exceeding 1500 μm in length. The number of peripheral cells in the vermiform phases is usually 22. The disc-shaped calotte and parapolar cells form the cephalic enlargement. The axial cell is cylindrical but is rounded anteriorly, and it extends forward to the base of propolar cells. Infusoriform embryos consist of 37 cells. In each of the four urn cells, there are the cell's own nucleus and one germinal cell with its own nucleus.

Dicyema clavatum sp. nov. is a relatively small sized dicyemid, infrequently reaching 1000 μm in length, and it is

the first mesozoan species described from O. minor. The number of peripheral cells in the vermiform phases is usually 22. The calotte is cap-shaped and smoothly rounded. The axial cell is enlarged and rounded in the calotte region, and it extends anteriorly to the base of the propolar cells. Uropolar cells occasionally become verruciform. Infusoriform embryos are composed of 39 cells. Each of the urn cells contains two nuclei of its own and one germinal cell with its own nucleus.

Further details relevant to the description of infusoriform embryos of Dicyema misakiense NOUVEL et NAKAO are provided and a note to Dicyema acuticephalum NOUVEL is given. The dicyemid fauna in the two species of octopuses is briefly discussed.

ACKNOWLEDGMENTS

We wish to express our sincere gratitude to Dr. Takasi Tokioka, the former Professor and Director of the Seto Marine Biological Laboratory, Faculty of Science, Kyoto University, for his active interest and valuable advice, and also to Ms. Kikuko Kurihara for her constant assistance in the course of this work. We are also indebted so much to Dr. Minoru Imajima, Head of the Zoology Division, Institute of Natural History, National Science Museum, for kindly allowing us to deposit syntypes of two new dicyemid species at the Institute.

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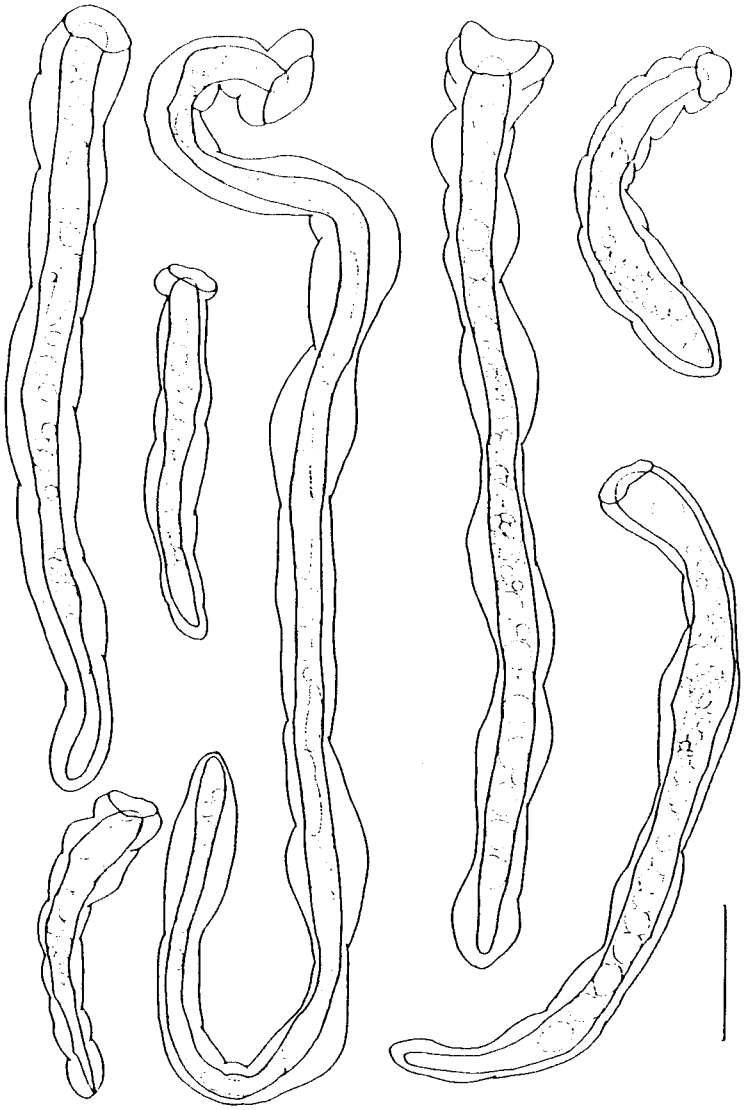

Fig. 1. Dicyema japonicum sp. nov. Four entire nematogens of various size are shown on the left and three rhombogens on the right. Note the characteristic shape of the head. Bar represents 100 µm. Drawn from specimens prepared from No. VU44 octopus.

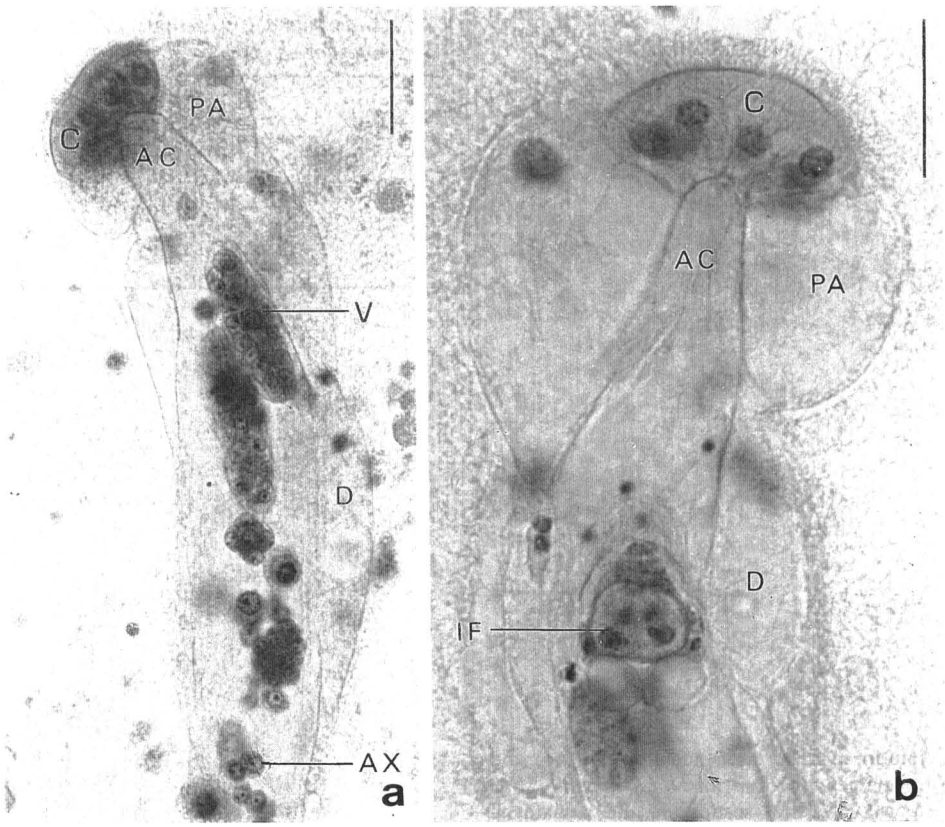

Fig. 2. Anterior part of a nematogen (a) and a rhombogen (b) of D. japonicum. Photographs were taken after alcholic Bouin fixation, which was followed by Ehrlich's acid hematoxylin and light green staining. Note that the calotte (c) is conspicuously stained and is covered with a dense array of cilia. AX, axoblast; AC, axial cell; D, diapolar cell; IF, infusoriform embryo; PA, parapolar cell; V, vermiform embryo. Bars represent 20 μm. (a) Prepared from No. VU103 octopus, (b) prepared from No. VU44 octopus.

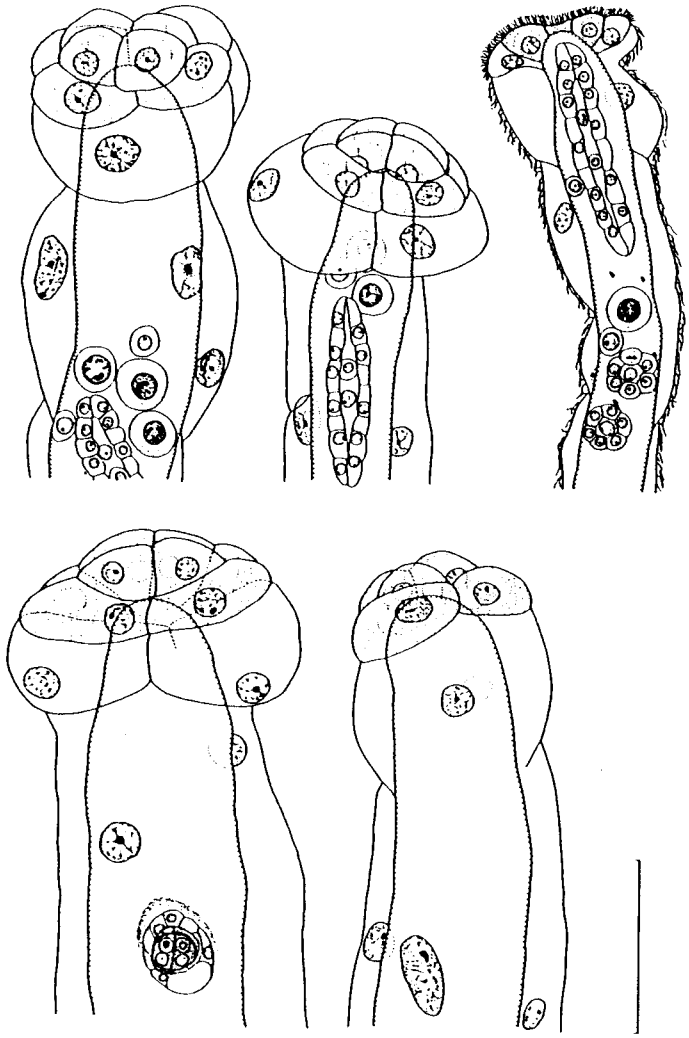

Fig. 3. Anterior part of three nematogens (top) and two rhombogens (bottom) of D. japonicum. Cilia are shown in optical section on the nematogen depicted on the far right. Bar represents 50 μm. Drawn from specimens prepared from No. VU103 octopus.

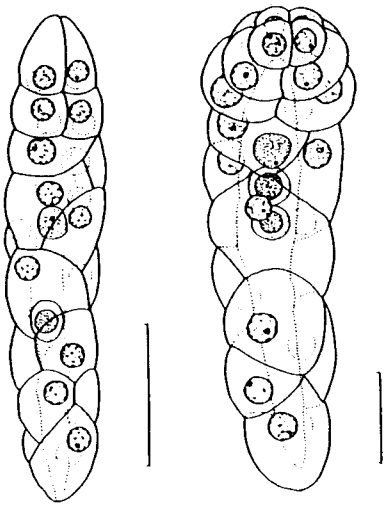

Fig. 4. A vermiform embryo eithin the axial cell (the cell outline is omitted) of a nematogen (on the left) and free-living vermiform larva (on the right) of D. japonicum. Bars represent 10 μm. Drawn from specimens prepared from No. VU103 octopus.

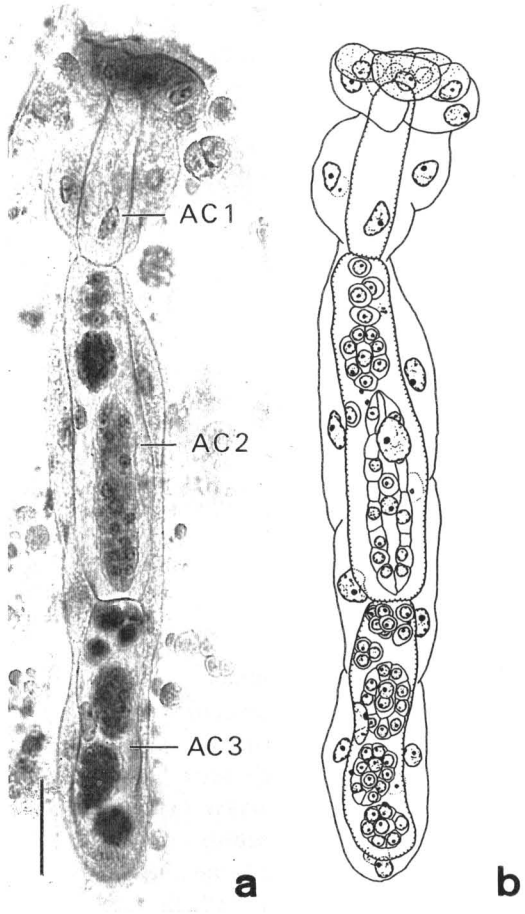

Fig. 5. A stem nematogen of D. japnicum with three axial cells. This individuals has vermiform embryos in both the second and third axial cell; in the first axial cell its nucleus is visible but no embryos are seen. Photograph (a) was taken after alcholic Bouin fixation, Ehrlich's acid hematoxylin and light green staining. The tracing (b) was drawn from the photograph. AC3, third axial cell. Bar represents 20 μm. Prepared from No. VU103 octopus.

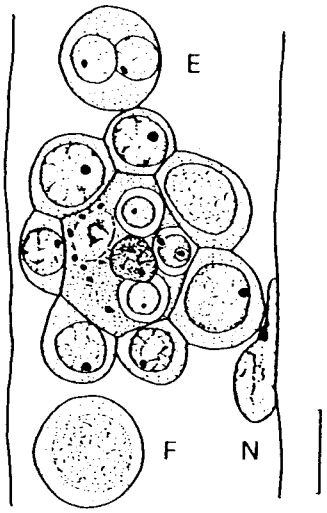

Fig. 6. Infusorigen of D. japonicum. Around the infusorigen, a two-pronuclei stage egg (E), a fertilized egg (F), and an axial cell nucleus (N) are found. Bar represents 10 μm. Drawn from a specimen prepared from No. VU103 octopus.

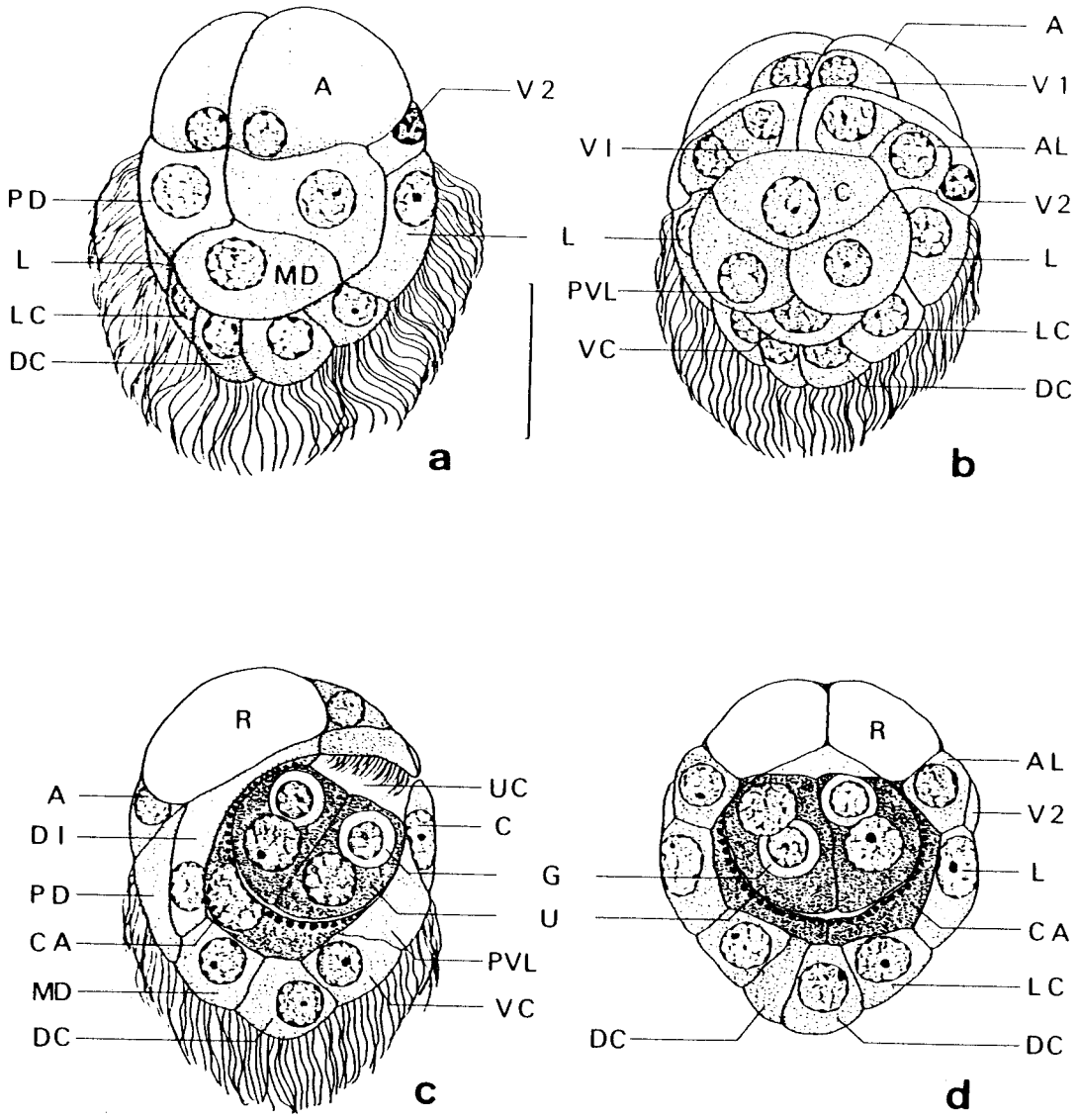

Fig. 7. Infusoriform embryo of D. japonicum. Dorsal view (a), ventral view (b), sagittal section (c), and horizontal section (d, cilia omitted) are shown. Emveloping cells are omitted. A, apical; AL, anterior lateral cell; C, courvercle cell; CA, capsule cell; DC, dorsal caudal cell; PVL, posteroventral lateral cell; R, refringent body; U, urn cell; UC, urn cavity; VC, ventral caudal cell; VI, ventral internal cell; V1, first ventral cell; V2, second ventral cell. Bar represents 10 μm. Drawn from specimens prepared from No. VU103 octopus.

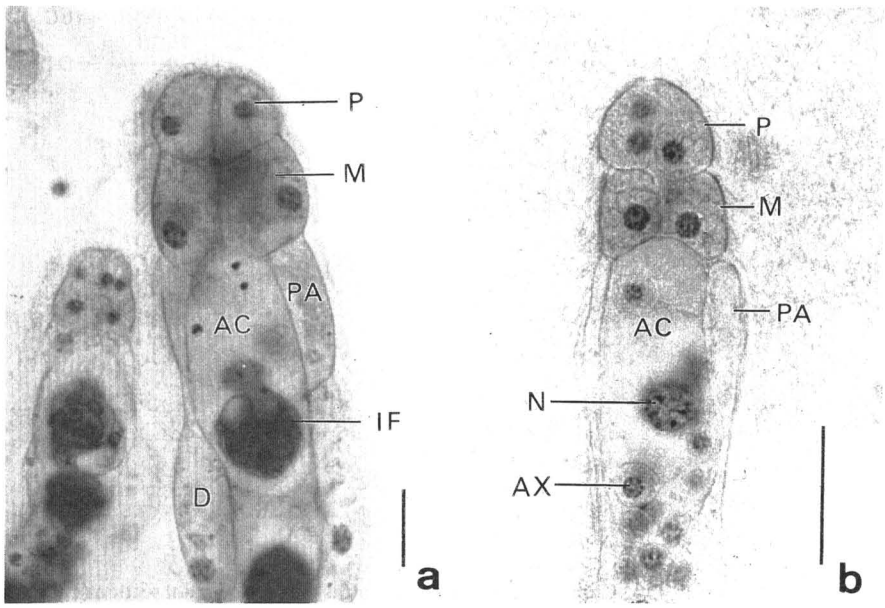

Fig. 8. Anterior part of two rhombogens of D. misakiense (a) and a nematogen of D. acuticephalum (b). Compare the head shape of these species with that of D. japonicum (shown in Fig. 2). Photographs were taken after alcholic Bouin fixation, Ehrlich's acid hematoxylin and light-green staining. AC, axial cell; AX, axoblast; D, diapolar cell; IF, infusoriform embryo; M, metapolar cell; N, axial cell nucleus; P, propolar cell; PA, parapolar cell. Bar represents 20 μm. (a) prepared from No. VU44 octopus, (b) prepared from No. VU40 octopus.

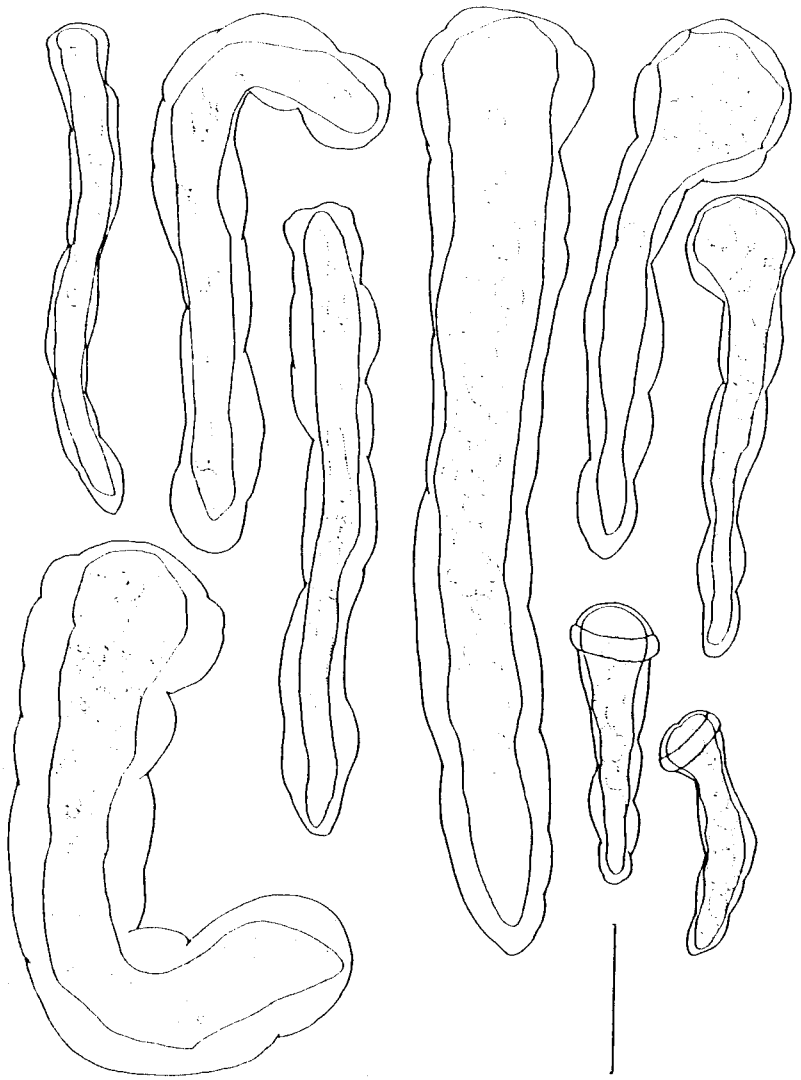

Fig. 9. D. clavatum sp. nov. Four entire nematogens of various sizes are shown on the left and five rhombogens on the right. Note the characteristic ballooning of anteior part of the axial cell. Bar represents 100 µm. Drawn from specimens from specimens prepared from No. MI95 octopus.

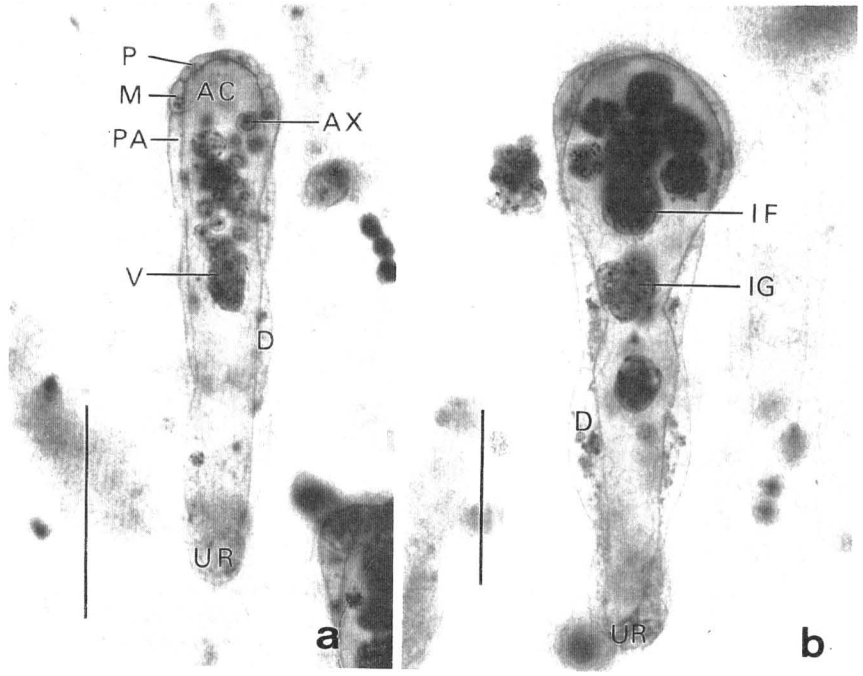

Fig. 10. Photographs of a nematogen (a) and a rhombogen (b) of D. clavatum, after the same procedure as used in the case of D. japonicum shown in Fig. 2. AX, axoblast; IG, infusorigen; M, metapolar cell; UR, uropolar cell. See the legend to Fig. 2 for other abbreviations. Bars represent 50 μm. Prepared from No. MI95 octopus.

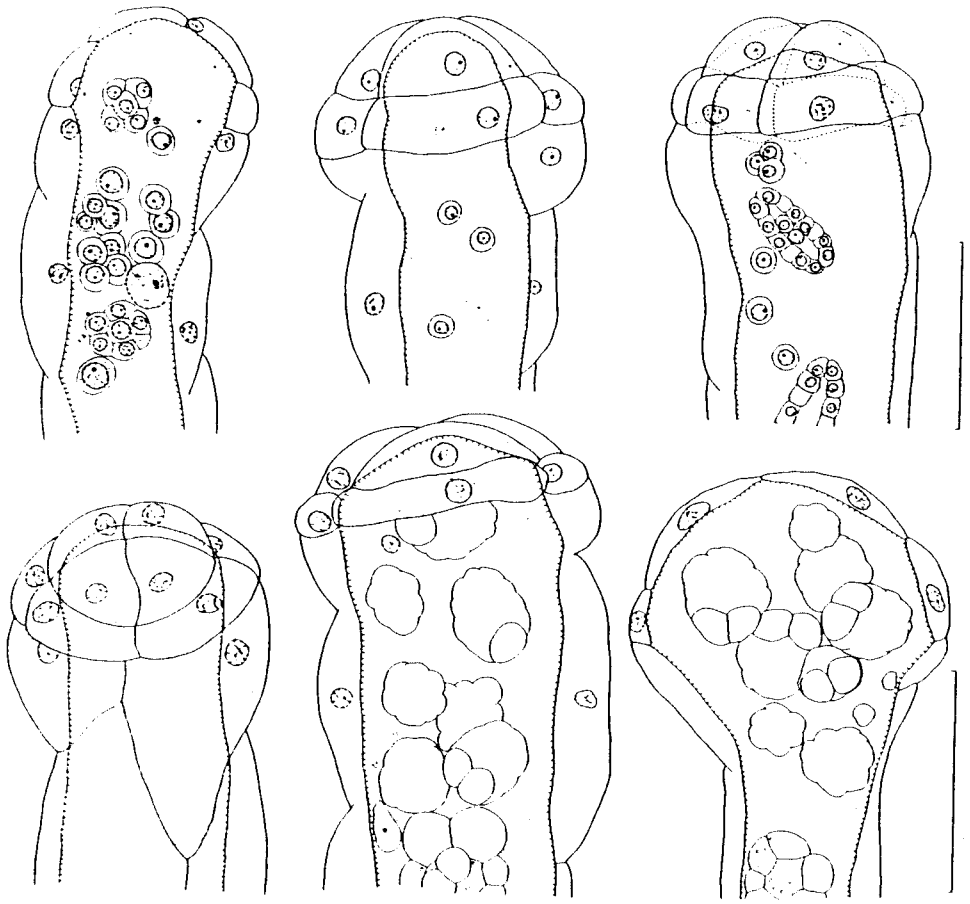

Fig. 11. Anterior part of three nematogens (top) and three rhombogens (bottom) of D. clavatum. The fat left nematogen and the far right rhombogen are depicted in optical section. Note the band -like appearance of the metapolar cells around the axial cell. Bars represent 50 μm. Drawn from specimens prepared from No. MI95 octopus.

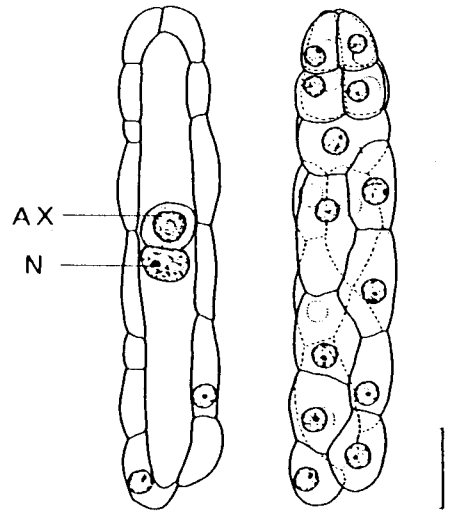

Fig. 12. Vermiform embryos of D. clavatum. The left embryo is depicted in the optical section to show one axoblast (AX) adjacent to the axial cell nucleus (N). The drawing of the right embryo shows an opposed-type arrangement of diapolar peripheral cells. Bar represents 10 μm. Drawn from specimens prepared from No. MI95 octopus.

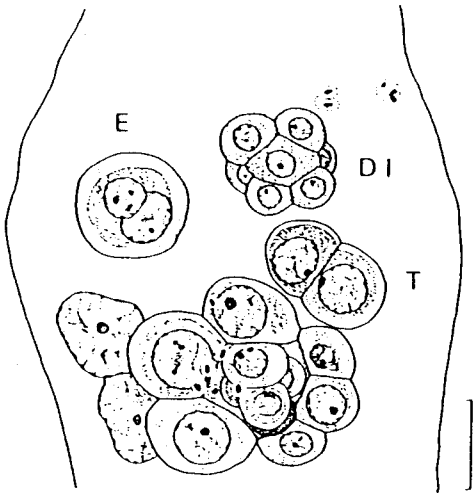

Fig. 13. Infusorigen of D. clavatum. Around the infusorigen, a fertilized egg at the two-nuclei stage (E), a developing infusoriform embryo (DI), and an embryo at the two-cell stage (T) are found. Bar represents 10 μm. Drawn from a specimen prepared from No. MI95 octopus.

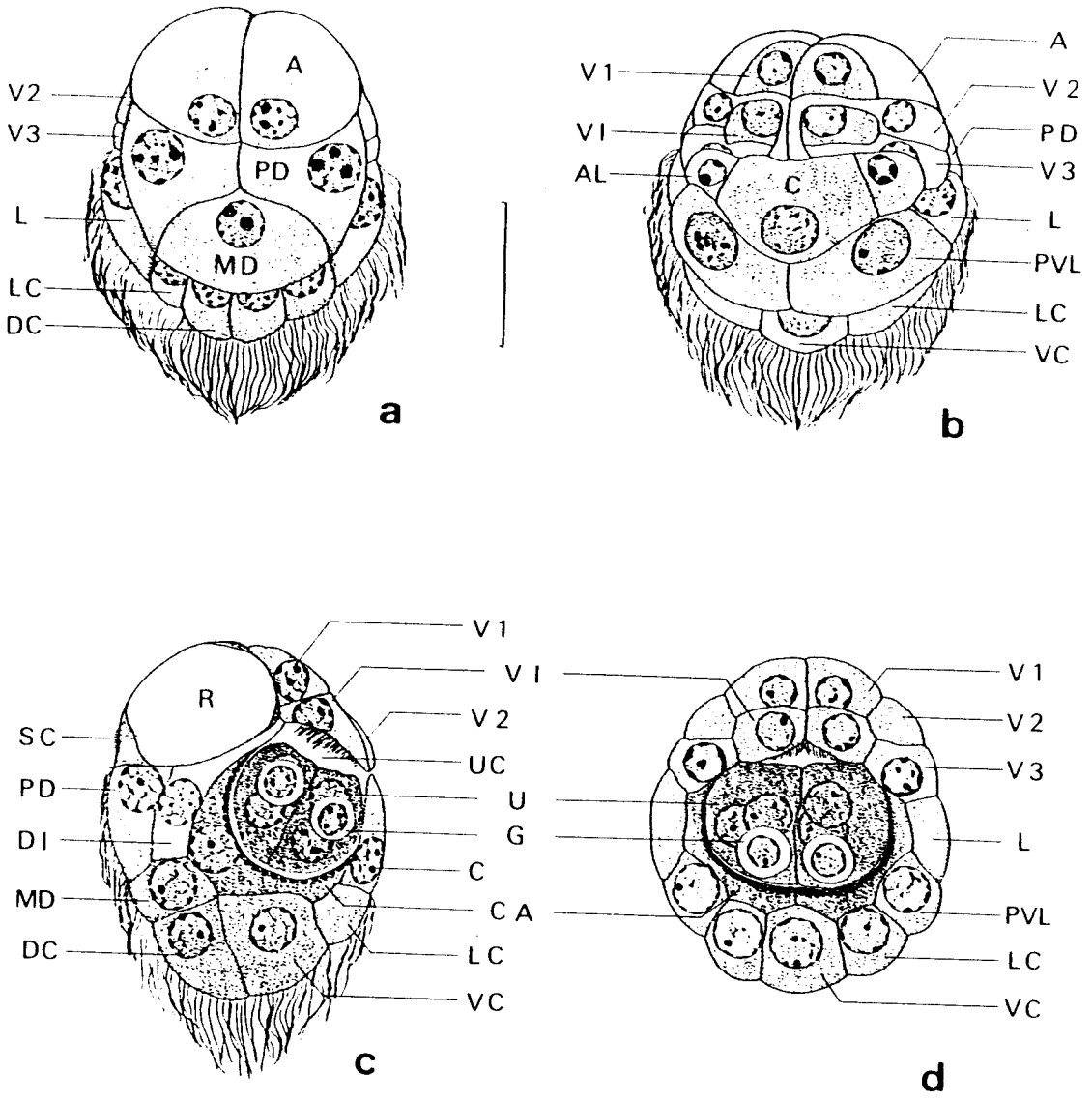

Fig. 14. Infusoriform embryo of D. clavatum. The dorsal view (a), the ventral view (b), a sagittal section (c), and a horizontal section (d, cilia omitted) are shown. Enveloping cells are omitted. SC, short cilia; V3, third ventral cell. See the legend to Fig. 7 for other abbreviations. Bar represents 10 μm. Drawn from specimens prepared from No. MI95 octopus.

TABLE 1. *Octopus vulgaris* and parasite dicyemids

Hosts						Dicyemids
No.	Dorsal mantle length (cm)	Body weight (g)	Sex	Source*	Date of examination	
VU12	7.2	350	M	1	06.21.1989	*D. acuticephalum* *D. japonicum* *D. misakiense*
VU98	9.5	865	F	1	07.14.1990	*D. japonicum* *D. misakiense* *D.* sp. (A)
VU5	7.4	270	F	1	06.14.1989	
VU16	5.4	78	M	2	07.06.1989	
VU17	5.7	123	M	2	07.06.1989	
VU18	6.5	148	M	2	07.06.1989	
VU20	7.5	—	F	5	07.24.1989	
VU28	9.5	—	M	3	08.04.1989	
VU32	15.2	—	M	4	08.10.1989	*D. japonicum*
VU41	12.5	740	F	2	10.20.1989	*D. misakiense*
VU43	8.2	528	M	6	10.27.1989	
VU44	9.7	738	M	6	10.27.1989	
VU45	6.9	460	M	1	12.01.1989	
VU83	13.5	465	M	1	05.11.1990	
VU101	5.3	96	M	2	07.21.1990	
VU102	6.6	218	F	2	07.21.1990	
VU103	7.9	—	F	5	07.30.1990	
VU104	7.8	508	M	2	05.08.1991	
VU3	8.2	260	M	1	04.26.1989	
VU4	11.4	770	F	1	05.30.1989	*D. acuticephalum*
VU40	9.7	750	F	2	09.06.1989	
VU105	9.2	550	M	1	05.27.1991	*D. misakiense*
VU15	4.0	70	F	2	07.06.1989	None

* Source numbers indicate the following:
(1) Commercially supplied from a fish market at Akashi (Hyogo Pref.) where fish dealers receive octopuses caught mainly inside Osaka Bay and/or in the Sea of Harima (the eastern area of the Inland Sea).
(2) Commercially supplied from a fish market at Shounai (Toyonaka, Osaka Pref.). Locations of these octopuses are presumably similar to those of octopuses bought at Akashi.
(3) Commercially supplied from a fish market at Shirahama (Wakayama Pref.). The octopus was probably caught inside Tanabe Bay where it faces the Pacific Ocean.
(4) Commercially supplied from a fish market at Sakaiminato (Tottori Pref.). The octopus was possibly obtained from the coast of the Sea of Japan near Sakaiminato.
(5) Caught by fishermen in Dozen, Oki Islands (Shimane Pref.), located in the Sea of Japan about 50 km to the north of Sakaiminato.
(6) Collected by the authors in Maizuru Bay (Kyoto Pref.) where it is open to the Sea of Japan.

TABLE 2. *Octopus minor* and parasite dicyemids

Hosts						Dicyemids
No.	Dorsal mantle length (cm)	Body weight (g)	Sex	Source*	Date of examination	
MI89	6.5	78	M	2	07.11.1990	*D. clavatum*
MI90	7.9	168	M	2	07.14.1990	*D. clavatum*
MI91	8.3	204	M	2	07.14.1990	*D.* sp. (B)
MI92	8.8	239	M	2	07.14.1990	*D. clavatum*
MI97	5.2	68	F	2	07.14.1990	*D. clavatum*
MI93	8.2	126	F	2	07.14.1990	*D. clavatum*
MI94	8.3	207	F	2	07.14.1990	*D. clavatum*
MI95	8.3	194	F	2	07.14.1990	*D. clavatum*
MI96	7.3	145	F	2	07.14.1990	*D. clavatum*
MI63	7.2	105	F	1	04.10.1990	*D.* sp. (B)
MI64	8.7	175	M	1	04.10.1990	*D.* sp. (B)
MI65	7.2	142	F	1	04.10.1990	*D.* sp. (B)
MI69	7.3	110	F	1	04.10.1990	*D.* sp. (B)
MI71	6.5	231	M	1	04.10.1990	*D.* sp. (B)
MI84	6.4	124	M	2	07.11.1990	*D.* sp. (C)
MI85	7.4	155	M	2	07.11.1990	*D.* sp. (C)
MI86	6.5	192	M	2	07.11.1990	*D.* sp. (C)
MI87	6.9	111	M	2	07.11.1990	*D.* sp. (C)
MI88	6.7	118	M	2	07.11.1990	*D.* sp. (C)

* See explanations for Table 1.

TABLE 3. Number of peripheral cells of *Dicyema japonicum* sp. nov.

No. of cells	Number of individuals		
	Vermiform embryos	Nematogens	Rhombogens
20	0	2	1
21	0	2	8
22	54	52	54

TABLE 4. Number of cells in infusoriform embryos and the nucleus number of urn cells in four dicyemid species

Species	No. of cells*	No. of nucleus of urn cells*
D. japonicum	37	1
D. misakiense	37	1
D. acuticephalum	37	2
D. clavatum	39	2

* These numbers were very constant in four species examined.

TABLE 5. Number of peripheral cells of *Dicyema misakiense*

No. of cells	Number of individuals		
	Vermiform embryos	Nematogens	Rhombogens
21	0	1	0
22	51	51	55

TABLE 6. Number of peripheral cells of *Dicyema acuticephalum*

No. of cells	Number of individuals		
	Vermiform embryos	Nematogens	Rhombogens
16	0	0	2
17	0	1	0
18	62	56	58
19	0	1	2

TABLE 7. Number of peripheral cells of *Dicyema clavatum* sp. nov.

No. of cells	Number of individuals		
	Vermiform embryos	Nematogens	Rhombogens
20	0	3	0
21	0	10	1
22	59	44	20

TABLE 8. Dicyemid species recorded as parasites of *Octopus vulgaris* in the literature

Species	Typical number of peripheral cells	Distribution	Reference
Dicyema acuticephalum	16–19	Japan	[2]
Dicyema aegira	22	Florida, U.S.A.	[5]
Dicyema bilobum	16–18	Florida, U.S.A.	[10]
Dicyema megalocephalum	16	West Africa	[12]
Dicyema misakiense	22	Japan	[1]
*Dicyema monodi**	16	West Africa	[12]
Dicyema paradoxum	28	Europe and West Africa	[9]
Dicyema typoides	18	Florida, U.S.A.	[11]
Dicyema typus	18–19	Europe	[2]
Dicyemennea lameerei	23	Europe	[13]
Conocyema polymorpha	12	Europe	[14]

* According to Bogolepova-Dobrokchotova [15], this species name is a synonym of *D. megalocephalum*.

CHAPTER II

Development of the Infusoriform Embryo of *Dicyema japonicum* (Mesozoa: Dicyemidae)

Abbreviations of cell names

A	apical cell	LC	lateral caudal cell
AL	anterior lateral cell	MD	median dorsal cell
C	couvercle cell	PD	paired dorsal cell
CA	capsule cell	PVL	posteroventral lateral cell
DC	dorsal caudal cell	U	urn cell
DI	dorsal internal cell	V1	first ventral cell
E	enveloping cell	V2	second ventral cell
G	germinal cell	VC	ventral caudal cell
L	lateral cell	VI	ventral internal cell

INTRODUCTION

The bodies of dicyemid mesozoans consist of only 20 to 40 cells and are organized in a very simple fashion (Nouvel, 1948; McConnaughey, 1951). Although Hyman (1940, 1956) considered dicyemids to be truly primitive multicellular animals, until some twenty years ago many zoologists regarded the simple body organization of dicyemids as the result of degeneration due to parasitism in the cephalopod kidney (Nouvel, 1948; McConnaughey, 1951; Stunkard, 1954; Ginetsinskaya, 1988). However, recent studies on the base compositions and sequences of their nucleic acids have suggested that dicyemids are somewhat closer to ciliate protozoans than to flatworms (Lapan and Morowitz, 1974; Hori and Osawa, 1987).

In any attempt to evaluate the phylogenetic position of an organism, a knowledge of the normal development of the organism can be crucial. In the case of dicyemids, such information may also attract the attention of developmental biologists because, in the animal kingdom, the development of dicyemids may represent one of the simplest patterns of cell differentiation that occurs during embryogenesis. Nevertheless, the development of the infusoriform embryo of dicyemids has been studied to only a very limited extent (McConnaughey, 1951; Sponholtz, 1964; Lapan and Morowitz, 1975) and the details of the cell lineage during embryogenesis remain to be determined.

In this report, we describe the pattern of cleavage and the cell lineage during the development of the infusoriform embryo of

Dicyema japonicum. In the complex life cycle of dicyemids, the vermiform embryo and the infusorigen each develop asexually from an axoblast (agamete) and the stem nematogen is believed to develop asexually from a germinal cell of the infusoriform embryo (McConnaughey, 1951; Lapan and Morowitz, 1975; Hochberg, 1982, 1983). The infusoriform embryo is the only form that develops from a fertilized egg and the development that we discuss herein is that of infusoriform embryos. In Dicyema japonicum, they are ultimately composed of 37 cells.

MATERIALS AND METHODS

Seventeen host octopuses, Octopus vulgaris, were purchased or collected personally in the western part of Japan. Although, in this region, four species of dicyemids are found in the kidneys of Octopus vulgaris (Furuya et al., 1992), only Dicyema japonicum Furuya et Tsuneki, 1992 was examined throughout this study. After the octopus had been sacrificed, its kidneys were taken out and smeared directly on glass slides. Smeared dicyemids were immediately fixed with Carnoy's fixative or alcoholic Bouin's solution (absolute ethanol saturated with picric acid: formalin: acetic acid, 15:5:1). Specimens fixed with Carnoy's fixative were stained with Feulgen's stain or by the PAS method and were poststained with Ehrlich's hematoxylin and light green. Some specimens were treated with saliva before the PAS staining. Specimens fixed with alcoholic Bouin's solution were stained with Ehrlich's hematoxylin and light green only. The embryos in the

axial cell of rhombogens were observed with the aid of a light microscope under an oil-immersion objective at a magnification of 2000 diameters. Blastomeres were identified by criteria such as position within the embryo, size of nucleus and cell, and stainability of nucleus and cell. By paying careful attention, we identified each swollen nucleus that was about to divide and each metaphase figure in terms of the blastomere that was going to divide and the resulting two daughter blastomeres. Each developing embryo with or without dividing blastomeres was sketched at three different optical depths and a three-dimensional diagram was reconstructed from these sketches. The fully formed embryo consisted of only 37 cells and special techniques such as injection of a tracer and videoscopy were not required for determination of the cell lineage. The early cleavages of Dicyema japonicum were spiral and, therefore, the terminology of blastomeres that is generally used for embryos with spiral cleavages was adopted in designating the blastomeres. The cells of the infusoriform were named according to the earlier authors (Nouvel, 1948; McConnaughey, 1951; Short and Damian, 1966; Ridley, 1969; Matsubara and Dudley, 1976).

RESULTS

In Dicyema japonicum, there is usually only one infusorigen, which is functionally a hermaphroditic gonad, and it is located in the center of the axial cell of a rhombogen (Fig. 1). A sperm enters the oocyte, which is located around the axial cell of the

infusorigen. Then the oocyte undergoes meiosis and produces the polar bodies, and the two-pronucleus stage follows (Figs. 2a-d). Fertilized eggs are about 12.3 μm in diameter. As development proceeds from the 2-cell to the 4-cell stage and beyond (Figs. 2e-h), the embryo leaves the infusorigen and moves toward the anterior or posterior end of the axial cell of the rhombogen. In large specimens, there are more than 20 embryos, including fully formed infusoriforms, in a single rhombogen. In some cases, therefore, nearly the entire series of developmental stages can be observed in a single fixed rhombogen.

The cleavage is holoblastic and early cleavages proceed spirally. The first cleavage is meridional and equal, and produces two blastomeres, AB and CD (Figs. 2e, 3a). The second cleavage is latitudinal and equal, and produces four blastomeres, A, B, C, and D (Figs. 2f, 3b). Blastomeres A and C are in contact with each other at the animal pole, and blastomeres B and D are in contact at the vegetal pole. The third cleavage is again equal and four blastomeres, 1a, 1b, 1c, and 1d, are formed at the animal hemisphere (Figs. 2g, 3c). When viewed from the animal pole, these blastomeres are organized spirally clockwise to their sister blastomeres (1A, 1B, 1C, 1D), which form the vegetal hemisphere. The 8-cell embryo, thus, consists of two tiers of four cells (quartet). The descendants of blastomeres A and 1B are destined to form the left side of the infusoriform and the descendants of blastomeres C and 1D form the right side of the embryo.

The fourth cleavage is unequal and results in the 16-cell embryo (Figs. 2i, 3d). Four blastomeres, 1a, 1b, 1c, and 1d,

divide and produce the mesomeres $1a^1$, $1b^1$, $1c^1$, and $1d^1$ at the animal pole, and the macromeres $1a^2$, $1b^2$, $1c^2$, and $1d^2$ in the equatorial region. These divisions are not typically spiral. Blastomeres $1a^1$, $1b^1$, $1c^1$, and $1d^1$ undergo no further division. Blastomeres $1a^1$ and $1c^1$ become the dorsal caudal cells (DC) of the left and right side of the embryo, respectively. Blastomeres $1b^1$ and $1d^1$ become the median dorsal cell (MD) and the ventral caudal cell (VC), respectively. Macromere $1d^2$ also does not divide further and ultimately becomes the couvercle cell (C). These three cells (MD, VC, C) are located in the midline of the embryo and, thus, are not paired. Among the other macromeres, $1a^2$ and $1c^2$ usually undergo no further divisions and become the lateral caudal cell (LC) of the left and right side, respectively. Macromere $1b^2$ subsequently divides once more (Fig. 3j). The resultant sister blastomeres gradually accumulate a refringent body in the cytoplasm and finally become the apical cells (A).

The fourth division in the vegetal hemisphere generates four sub-macromeres, 2a, 2b, 2c, and 2d, in the subequatorial region, and four micromeres, 2A, 2B, 2C, and 2D, at the vegetal pole. Micromeres 2A, 2B, 2C, and 2D do not divide further and they ultimately become the germinal cells (G). After the fourth division, the cleavages are not synchronized among the blastomeres (Figs. 2j-n, 3e-l, 4).

The 20-cell stage is achieved by the unequal division of blastomeres 2a, 2b, 2c, and 2d (Fig. 3e). When the embryo is viewed from the vegetal pole, the daughter blastomeres $2a^1$, $2b^1$, $2c^1$, and $2d^1$ occupy the left side of $2a^2$, $2b^2$, $2c^2$, and $2d^2$,

respectively. At around this stage, the four micromeres 2A, 2B, 2C, and 2D (the presumptive germinal cells) are incorporated into the inside of the embryo as the other blastomeres grow and rearrange themselves. Blastomeres $2a^1$ and $2d^1$ undergo no further divisions and eventually they become the posteroventral lateral cells (PVL) of the left and right side, respectively.

The cleavage pattern beyond the 20-cell stage is not spiral but bilateral. Beyond this stage, the order of divisions of blastomeres is not necessarily identical among developing embryos and the subsequent developmental stages, such as the 24-cell stage and so on, become increasingly less well defined. For example, in the embryo shown in Figure 3f (23-cell stage), blastomere $2c^{22}$ has already divided into its daughter cells while, in the embryo shown in Figure 3h (24-cell stage), the same blastomere is still intact.

Blastomeres $2b^1$ and $2c^1$ usually divide once more and generate the paired dorsal cells (PD) and lateral cells (L) on the left and right side, respectively. In some individuals, however, these blastomeres ($2b^1$ and $2c^1$) do not divide further and simply become the paired dorsal cells. In these embryos, macromeres $1a^2$ and $1c^2$ divide once more and produce the lateral cells and the lateral caudal cells. In every case, the embryos become slightly oval in shape.

Blastomeres $2a^2$, $2b^2$, $2c^2$, and $2d^2$ exhibit complex patterns of cleavages. Blastomere $2a^2$ usually undergoes extremely unequal division (Fig. 2k); the much smaller sister blastomere (not named here) becomes pycnotic and is destined sooner or later to degenerate. The much larger sister blastomere soon divides again

and produces $2a^{21}$ and $2a^{22}$. Blastomere $2a^{21}$ becomes the capsule cell (CA), while blastomere $2a^{22}$ divides once more and produces the anterior lateral cell (AL; $2a^{221}$) and the second ventral cell (V2; $2a^{222}$). The capsule cell has a large nucleus and later accumulates PAS-positive granules in the cytoplasm. These granules are PAS-positive even after the saliva-test is applied. The second ventral cell later extends a long cytoplasmic process medially. The cleavage pattern and cell lineage of blastomere $2d^2$ are the same as those of blastomere $2a^2$, although $2d^2$ produces the cells that occupy the right side of the embryo.

Blastomere $2b^2$ first divides into $2b^{21}$ and $2b^{22}$, and blastomere $2b^{22}$ soon divides again. The resultant blastomere $2b^{221}$ becomes flattened (Fig. 4d); it covers the anterior region of the embryo and eventually it is transformed into the enveloping cell (E). The sister blastomere $2b^{222}$ becomes the first ventral cell (V1). The blastomeres generated from $2b^{21}$ are gradually incorporated into the inside of the embryo as the blastomeres derived from the animal hemisphere grow and rearrange themselves. Blastomere $2b^{211}$ then divides (Fig. 2m) to produce blastomeres $2b^{2111}$ and $2b^{2112}$. Blastomere $2b^{2111}$ becomes transparent apart from its nucleus and it is ultimately designated the dorsal internal cell (DI). Blastomere $2b^{2112}$ eventualy projects cilia into a small cavity, the urn cavity, and becomes the ventral internal cell (VI). The urn cavity is a cleft formed between the ventral internal cells and the urn that is composed of four urn cells. Blastomere $2b^{212}$ finally divides and produces two urn cells (U) of the left side. The cleavage pattern and cell lineage of blastomere $2c^2$ are the same as those

of blastomere $2b^2$: the descendants of $2c^2$ ultimately contribute to the right side of the embryo's body. The urn itself apparently rotates as a mass and, thus, it cannot be determined for each urn cell whether it was originally the right one or left one.

After all cells of the embryo have been laid down, one germinal cell is incorporated into the cytoplasm of each of the four urn cells (Fig. 5d). In Dicyema japonicum, the nucleus of each urn cell does not divide and each urn cell contains only one nucleus throughout. As the infusoriform matures, the nuclei of most of the cells tend to become pycnotic. Nuclear pycnosis takes place first in the second ventral cell. However, the nuclei of the germinal cells and the urn cells do not become pycnotic, even in the mature infusoriform.

The cell lineage of the infusoriform embryo of Dicyema japonicum is summarized in Figure 6. Blastomeres A and D follow exactly the same pattern of cleavages. Blastomeres B and C follow a very similar pattern of cleavages. A difference is found only in blastomeres $1b^2$ and $1c^2$; the former divides once to produce the apical cells but the latter does not. Even in the A and D series, however, corresponding blastomeres do not necessarily produce the same types of cell. For example, blastomere $1a^1$ becomes the dorsal caudal cell of the left side, while blastomere $1d^1$ produces the ventral caudal cell in the midline. Ultimately, four to eight rounds of cell division take place, excluding extremely unequal divisions, until each cell is established (Table I). Germinal cells are one type of cell that is determined early in embryogenesis. The cell lineage is

invariant, apart from the derivation of the lateral cell. As mentioned above, it is usually derived from $2b^1$ and $2c^1$ (see Fig. 6), but in some embryos it is derived from $1a^2$ and $1c^2$. Extremely unequal divisions, accompanied by degeneration of the much smaller daughter blastomeres, usually occur in blastomeres $2a^2$ and $2d^2$. However, it remains to be determined whether the unequal divisions occur consistently in these blastomeres in every embryo, and whether such divisions never occur in the $2b^2$ and $2c^2$ series.

The fully formed embryo consists of 37 cells (Fig. 5). Two enveloping cells (E) are flat and enclose the anterior half of the embryo; these cells are not depicted in Figure 5. Two apical cells (A) are completely enclosed by these cells and each contains one large refringent body in the cytoplasm. The nucleus is in the dorsocaudal part of each apical cell. The refringent body is known to contain magnesium inositol hexaphosphate, at least in Dicyema typus (Lapan, 1975). The external cells that are distinctly ciliated are as follows: two paired dorsal cells (PD), a median dorsal cell (MD), two dorsal caudal cells (DC), two lateral caudal cells (LC), a ventral caudal cell (VC), two lateral cells (L), and two posteroventral lateral cells (PVL). The cells forming the ventral surface of the embryo do not have cilia. They include two first ventral cells (V1), two second ventral cells (V2), two anterior lateral cells (AL), and a couvercle cell (C) which covers up the urn. Five cell types constitute the interior of the embryo. Two ventral internal cells (VI) have cilia that project into the urn cavity. Two dorsal internal cells (DI) and two capsule cells (CA) are not

ciliated. The dorsal internal cells were once called glycogen cells (McConnaughey, 1951), but they appear to be PAS-negative. By contrast, the capsule cells have PAS-positive granules in their cytoplasm. Each of the four urn cells encloses a germinal cell in its cytoplasm. The body length, excluding cilia, of the fully formed embryo is about 24 μm and the body width is about 19 um.

DISCUSSION

The processes of fertilization and extrusion of the polar body in dicyemid mesozoans were studied in detail by Short and Damian (1967). However, the development of the infusoriform embryo, which is the only organism that is produced directly after fertilization, has only been studied to a limited extent (McConnaughey, 1951; Sponholtz, 1964; Lapan and Morowitz, 1975). The pattern of development in Dicyema japonicum, which is described herein in detail, is very different from that briefly described for Dicyemennea adscita and some other species by McConnaughey (1951) and from the cursory depiction in the case of unspecified species by Lapan and Morowitz (1975), although these earlier reports indicated that the early cleavages followed a spiral pattern, as we observed in this study. A spiral pattern of early cleavages appears to be universal among dicyemids.

Spiral cleavage in dicyemids is reminiscent of that in flatworms (Platyhelminthes) and this similarity may be used as an argument for a phylogenetic relationship between dicyemids and

flatworms. In acoels, however, cleavages proceed by duets and small blastomeres are not produced at the vegetal pole (Apelt, 1969; Henley, 1974). In polyclads, early cleavages proceed by quartets as in dicyemids, but four small macromeres produced at the vegetal pole of the polyclad embryo are later absorbed during embryogenesis (Hyman, 1951; Kato and Minegishi, 1983). Details of developmental patterns, thus, are different between dicyemids and flatworms. In sponges (Porifera), the early cleavages are usually radial and, in coelenterates (Cnidaria), it is radial, bilateral, or partly spiral (Uchida and Yamada, 1983). The cleavage patterns of these primitive invertebrates are so diverse that a similarity in cleavage pattern per se may not necessarily reflect a phylogenetic relationship between the organisms concerned. Development of placozoans, Trichoplax adhaerens, has been only partly described (Grell, 1972), and development of fertilized eggs of the Orthonectida has never been described. Detailed comparative studies on the development of these "mesozoan" animals are necessary if we are to gain any insight into details of the evolution of these animals.

In the infusoriform embryos of dicyemids, there is no germ layer and groups of cells are roughly distinguished only as outer cells and inner cells. The outer cells, which occupy the dorsal and caudal surfaces of the embryo, are ciliated and are derived from the blastomeres of the animal hemisphere of the embryo, and the inner cells are derived from the blastomeres of the vegetal hemisphere. The innermost germinal cells are derived from the cells that form the vegetal pole. These processes of cellular rearrangement are observed in many other groups of animals and

appear to represent the basic pattern of the early development of animals. It is also apparent that the outer ciliated cells differentiate much earlier than the inner cells, with the exception of the germinal cells (Fig. 6 and Table I). In infusoriform embryos, the cells of the vegetal hemisphere are apparently incorporated passively into the interior of the embryo as the cells of the animal hemisphere proliferate. This type of development is similar to the epiboly seen in the stereoblastulae of some invertebrates including flatworms (Hyman, 1951; Henley, 1974). In dicyemids, a cavity called the urn cavity appears between the ventral internal cells and the urn, but this slit-like space is certainly formed secondarily and cannot be taken to represent a blastocoel.

In many dicyemid species, including Dicyema japonicum, the infusoriform finally consists of 37 cells, but in some species belonging to the genus Dicyema or Dicyemennea the infusoriforms consist of 39 cells (Short, 1971). In these latter species, there is a pair of third ventral cells in addition to the standard 37 cells. In peculiar cases such as in Dicyema knoxi, the infusoriform is composed of 37 cells, but there is a pair of postcapsular cells instead of a pair of anterior lateral cells (Short, 1971). In these cases, clearly, the last part of the cell lineage of the embryos is different from that of Dicyema japonicum. In Dicyema japonicum, the two blastomeres $2a^2$ and $2d^2$ usually undergo extremely unequal divisions and the resultant, much smaller daughter cells degenerate without contributing to the formation of the embryo. In his short description of the development of infusoriforms, McConnaughey (1951) noted the

occurrence of chromosome elimination during embryogenesis. In Dicyema japonicum, at least, what takes place is not chromosome elimination, but an unequal division that results in the pycnotic degeneration of the smaller blastomere. We can offer no explanation, at present, for the production of blastomeres that are destined to die. Programmed cell death is also noted in the embryogenesis of the nematode, Caenorhabditis elegans (Sulston et al., 1983).

In summary, the infusoriforms of dicyemids are consistently composed of only 37 or 39 cells. These cells, with more or less clear evidence of specific differentiation, such as dense cilia, PAS-positive granules, refringent bodies, and so on, are produced after only a vey few rounds of cell division. The development of the infusoriform embryos of dicyemids appears to be the simplest type of development seen in the animal kingdom. Thus, these infusoriform embryos might be useful as the simplest model system for the study of cell differentiation and morphogenesis in animals, especially if a method for culture of these embryos outside the axial cell becomes available and mutants can be generated.

SUMMARY.

The cleavage pattern and cell lineage of the infusoriform embryo of the dicyemid mesozoan Dicyema japonicum were studied in fixed material with the aid of a light microscope. The early cleavages are holoblastic and spiral. At the 16-cell stage, the

animal pole consists of four mesomeres, the equatorial region consists of four macromeres with four alternating sub-macromeres, and the vegetal pole is composed of four micromeres. At around the 20- to 24-cell stage, cleavage becomes asynchronous and its pattern changes from spiral to bilateral. The four micromeres, namely, the presumptive germinal cells, do not divide further and are finally incorporated into the cytoplasm of four urn cells, which are generated after divisions of the sub-macromeres. The blastomeres situated in the animal hemisphere give rise to ciliated cells which cover the posterior part of the embryo. Two blastomeres ($2a^2$ and $2d^2$) undergo extremely unequal divisions and the much smaller sister blastomeres degenerate and ultimately disappear during embryogenesis. The fully formed embryo consists of 37 cells. These cells are produced after only four to eight rounds of cell division. The cell lineage appears to be invariant among embryos, apart from the derivation of the lateral cells.

Acknowledgments

We would like to express our sincere thanks to Ms. Kikuko Kurihara for her valuable assistance during the course of preparation of the manuscript.

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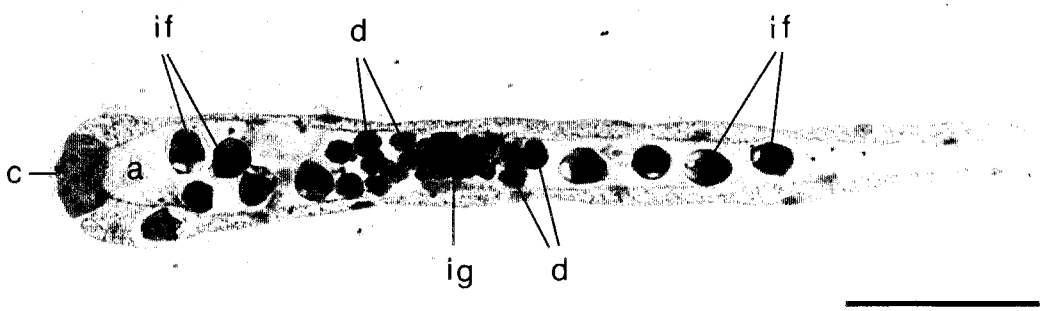

Figure 1. Light micrograph of a rhombogen of Dicyema japonicum. a, axial cell; c, calotte; d, developing infusoriform; if, infusoriform embryo; ig, infusorigen (hermaphroditic gonad). Scale bar represents 100 µm.

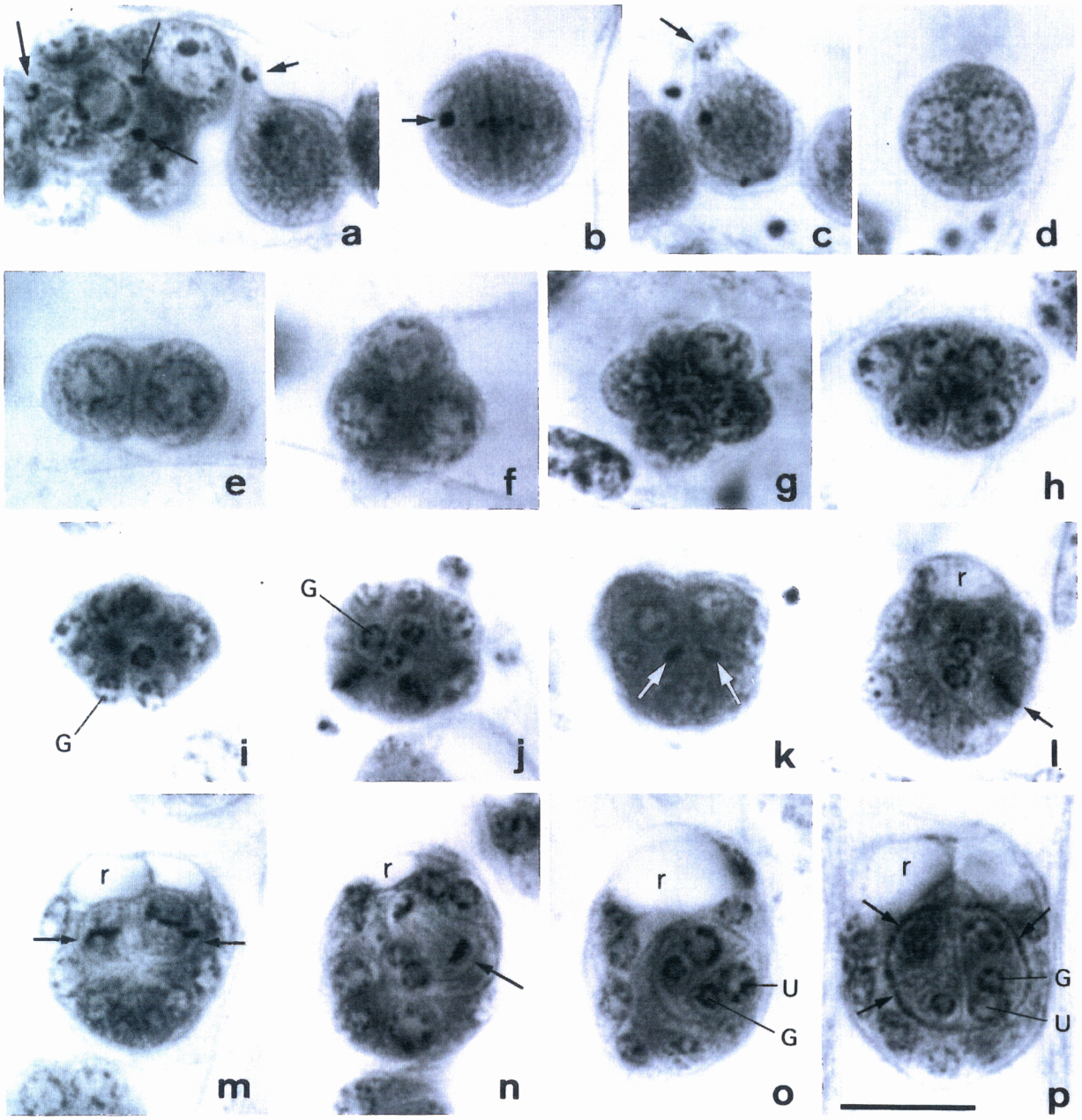

Figure 2. Light micrographs of eggs, developing infusoriform embryos, and fully formed infusoriform embryos of Dicyema japonicum. Photographs were taken at magnifications of 2000 diameters under an oil-immersion objective. Scale bar represents 10 µm. **a.** An infusorigen (left) and an oocyte undergoing meiosis (right). The short arrow indicates the first polar body

and the long arrows indicate spermatozoa. **b**. An oocyte undergoing the second meiotic division. The arrow indicates a spermatozoon. **c**. An oocyte finishing meiosis (center). The arrow indicates the second polar body. **d**. A fertilized egg at the 2-pronucleus stage. **e**. 2-cell embryo. **f**. 4-cell embryo. One blastomere is out of focus. **g**. 8-cell embryo. An axial cell nucleus is seen in the lower left corner. **h**. 12-cell embryo (optical section). **i**. 16-cell embryo (optical section). **j**. 24-cell embryo (optical section). **k**. 29-cell embryo (ventral view). The arrows indicate degenerating cells produced after extremely unequal divisions. **l**. 29-cell embryo (sagittal optical section). The arrow indicates a metaphase figure of $2d^2$. Two small nuclei in the center are those of germinal cells. r, Refringent body. **m**. 33-cell embryo (horizontal optical section). Arrows indicate metaphase figures of $2b^{211}$ and $2c^{211}$. r, Refringent body. **n**. 36-cell embryo (sagittal optical section). The arrow indicates a telophase figure of $2c^{212}$. r, refringent body. **o**. Fully formed embryo (sagittal optical section). r, refringent body. **p**. Fully formed embryo (horizontal optical section). Arrows indicate granules in capsule cells. r, refringent body.

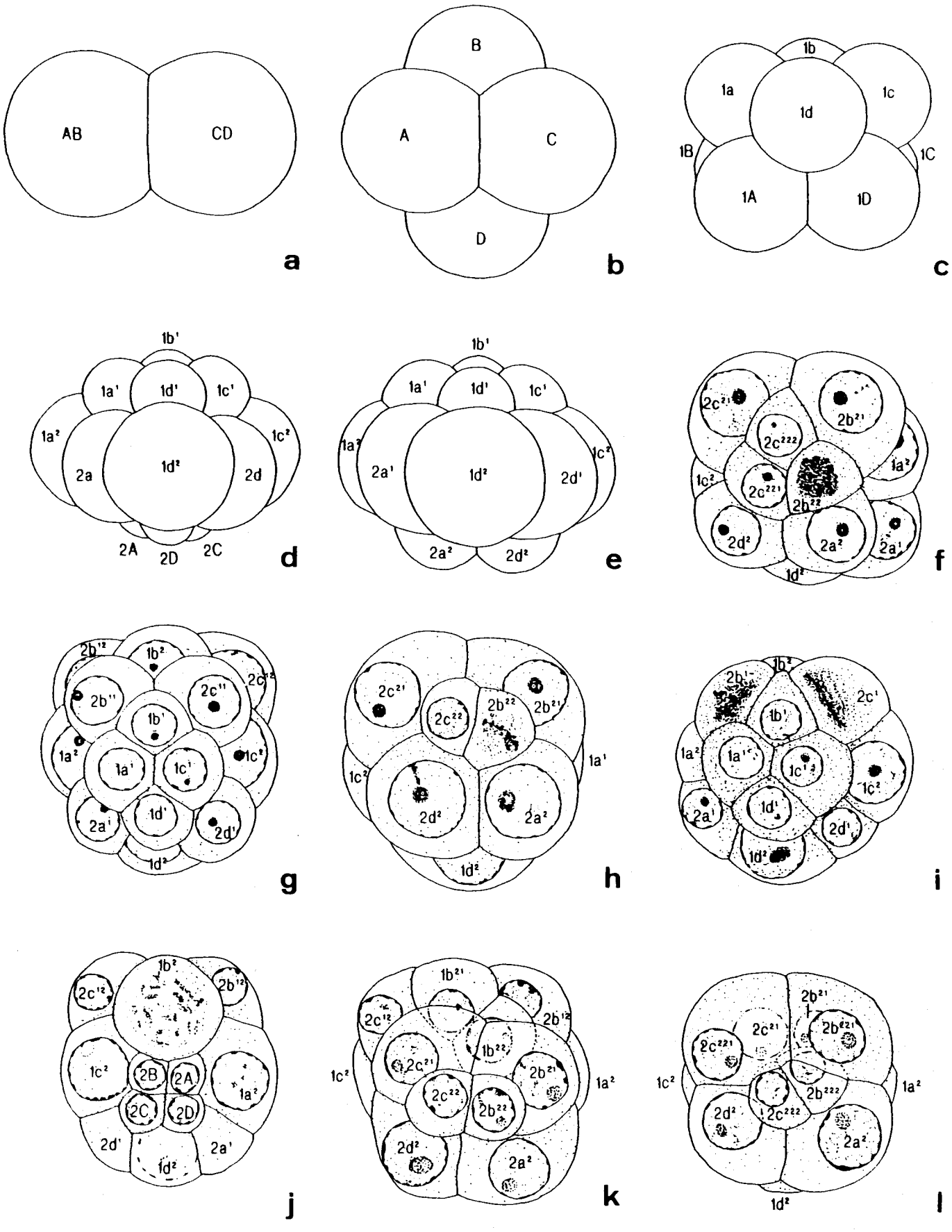
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Figure 3

Figure 3. Sketches of early embryos. Blastomeres are named according to the notation system for spiral cleavages. Scale bar = 10 μm. **a**. 2-cell stage (from the animal pole). **b**. 4-cell stage (from the animal pole). **c**. 8-cell stage (lateral view). **d**. 16-cell stage (lateral view). **e**. 20-cell stage (lateral view). Blastomeres 2A to 2D have been incorporated inside the embryo and, thus, they are not seen from outside. **f**. 23-cell stage (ventral view). Note a prophase figure in $2b^{22}$. **g**. 24-cell stage (dorsal view). **h**. 24-cell stage (ventral view). Note a metaphase figure in $2b^{22}$. **i**. 24-cell stage (dorsal view). Note prophase to metaphase figures in $2b^{1}$ and $2c^{1}$. **j**. 25-cell stage (horizontal optical section). Note an early prophase figure in $1b^{2}$. **k**. 25-cell stage (ventral view). **l**. 26-cell stage (ventral view).

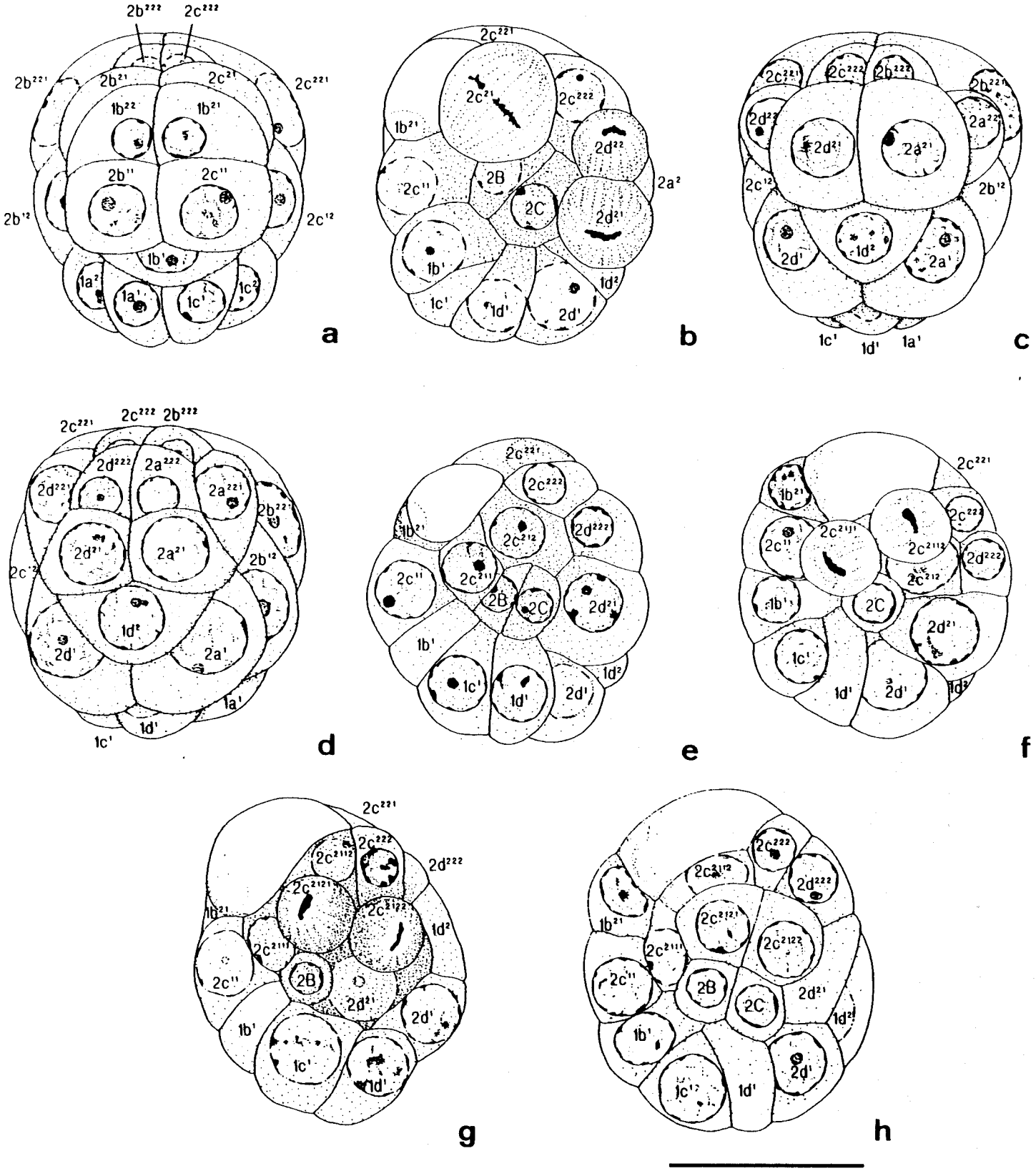

Figure 4

Figure 4. Sketches of late embryos. Scale bar = 10 μm. **a.** 29-cell stage (dorsal view). **b.** 29-cell stage (sagittal optical section). Note a metaphase figure in $2c^{21}$ and a telophase figure in $2d^{2}$. **c.** 29-cell stage (ventral view). **d.** 33-cell stage (ventral view). Large capsule cells ($2a^{21}$ and $2d^{21}$) are still situated on the ventral surface. **e.** 33-cell stage (sagittal optical section). **f.** 33-cell stage (sagittal optical section). Note a telophase figure in $2c^{211}$ which is dividing to produce dorsal and ventral internal cells ($2c^{2111}$ and $2c^{2112}$). **g.** 36-cell stage (sagittal optical section). Note a telophase figure in $2c^{212}$ which is dividing to produce two urn cells ($2d^{2121}$ and $2d^{2122}$). **h.** Early 37-cell stage (sagittal optical section). Cell divisions have been completed, but germinal cells (2B and 2C) have not yet been incorporated into the cytoplasm of urn cells ($2c^{2121}$ and $2c^{2122}$).

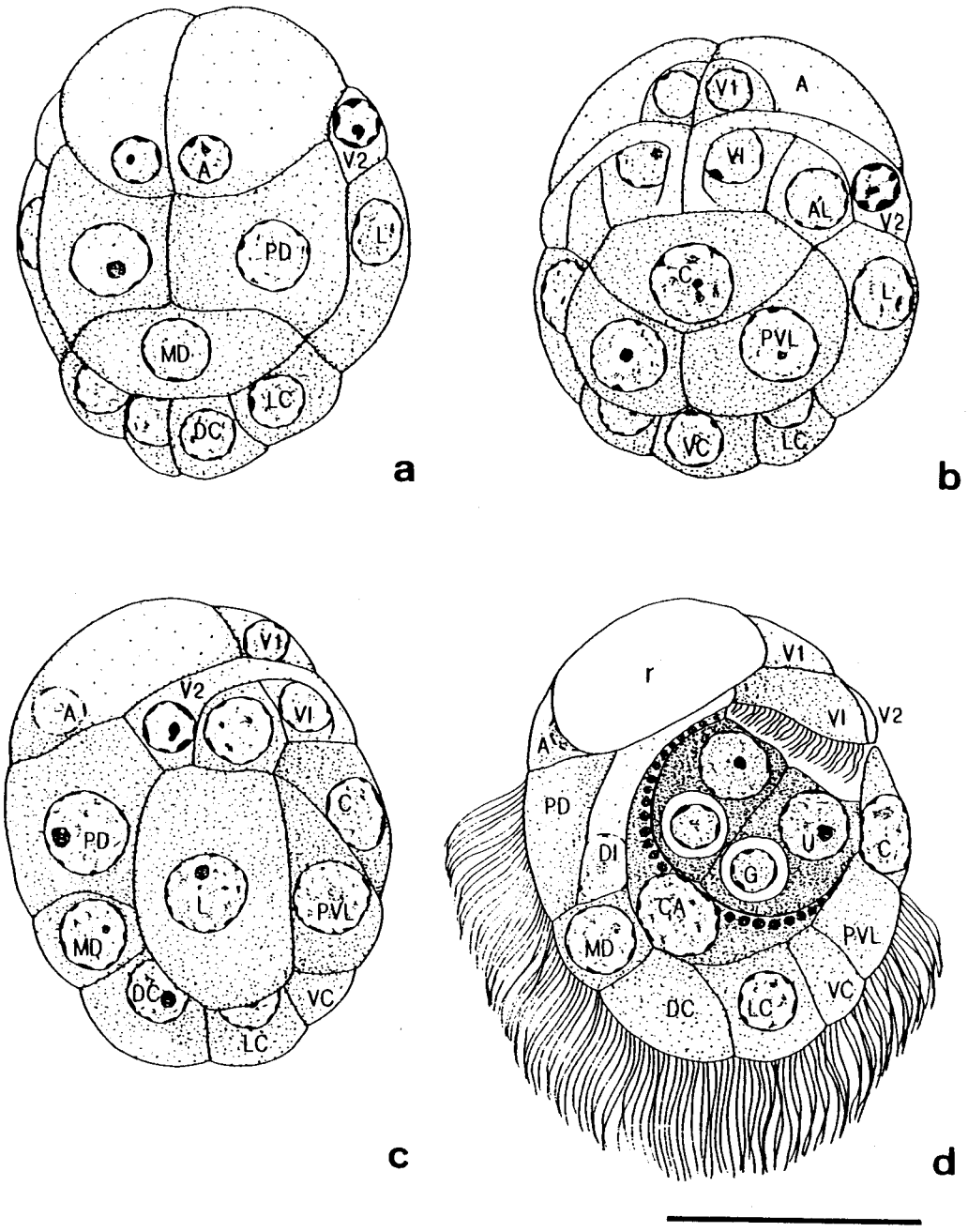

Figure 5. Sketches of a fully formed infusoriform embryo. Scale bar = 10 µm. **a**. Dorsal view. **b**. Ventral view. **c**. Lateral view. **d**. Sagittal optical section. Enveloping cells are not depicted. Cilia are omitted in **a**, **b**, and **c**. r, refringent body.

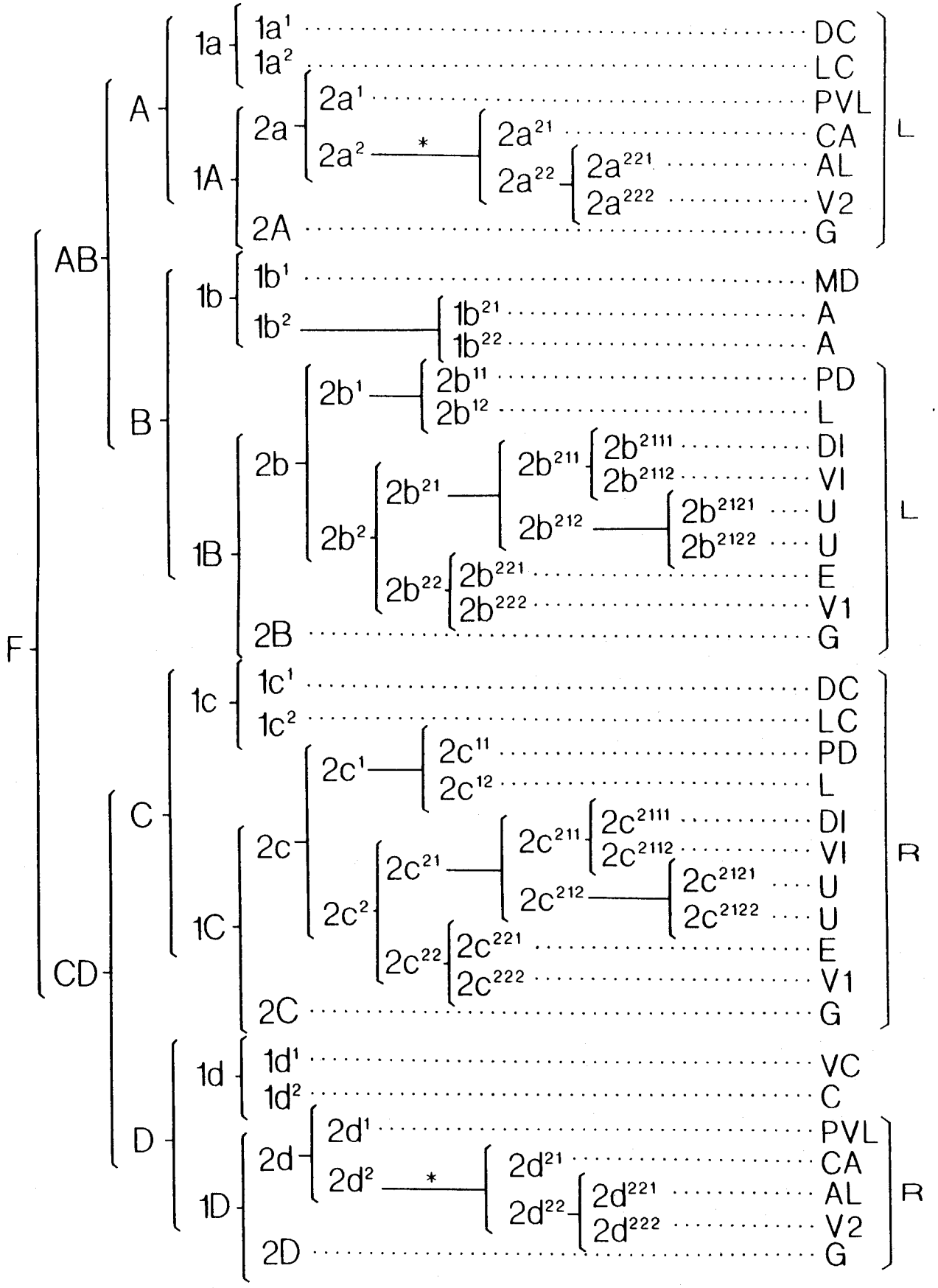
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Figure 6

Figure 6. Cell lineage of the infusoriform embryo of Dicyema japonicum. Blastomeres are named according to the notation system generally used for spiral cleavage. F implies a fertilized egg. L implies the left side of the embryo and R implies the right side. See the text for explanations of abbreviations in the right column. Blastomeres $2a^2$ and $2d^2$ usually undergo extremely unequal divisions at the points marked by asterisks. The much smaller daughter blastomeres degenerate and do not contribute to the formation of the embryo. In some embryos, $1a^2$ and $1c^2$ divide once more and produce lateral cells and lateral caudal cells. In these embryos, $2b^1$ and $2c^1$ do not divide further and simply become paired dorsal cells.

Table I

The number of cleavage divisions that precede formation of each type of cell.*

Number of divisions	Type of cell
4	Dorsal caudal cells (DC), lateral caudal cells (LC), median dorsal cell (MD), ventral caudal cell (VC), couvercle cell (C), germinal cells (G)
5	Posteroventral lateral cells (PVL), apical cells (A), paired dorsal cells (PD), lateral cells (L)
6	Capsule cells (CA)
7	Anterior lateral cells (AL), first ventral cells (V1), second ventral cells (V2), enveloping cells (E)
8	Dorsal internal cells (DI), ventral internal cells (VI), urn cells (U)

* Extremely unequal divisions are not counted. In some embryos, paired dorsal cells are formed after 4 rounds of division and lateral caudal cells after 5 rounds of division (see text).

CHAPTER III

The Development of the Hermaphroditic Gonad in Four Species of Dicyemid Mesozoans

INTRODUCTION

Dicyemid mesozoans are found in the renal sac of benthic cephalopod molluscs. The bodies of dicyemids consist of only 20 to 40 cells and they are organized very simply [12, 14]. It has long been debated whether dicyemids are truly primitive multicellular animals [2, 7, 8, 11, 17], or whether they are actually organisms that have degenerated as a result of parasitism [5, 12, 14, 19].

As well known, two kinds of adult forms, nematogens and rhombogens, are found in dicyemids. Asexual reproduction occurs within the axial cell of a nematogen, while sexual reproduction takes place within the axial cell of a rhombogen. The features of the sexual reproduction are unique [12, 15]. A hermaphroditic gonad, which is called an infusorigen, is formed within the axial cell of a rhombogen, and fertilization occurs around the infusorigen. The zygote undergoes cleavages and develops into an infusoriform larva within the axial cell. The process of the fertilization and embryogenesis of infusoriforms have been described in some detail [1, 3, 18], but the development of infusorigens has been studied only sporadically [10, 12, 14]. No research on the patterns of development of infusorigens has been performed from a systematic perspective. We examined the development of the infusorigens of Dicyema orientale, D. acuticephalum, D. japonicum, and D. misakiense, and found three different types of cell lineage. In this report, these three different cell lineages that can be followed during the development of infusorigens are described. In addition, we

provide an estimate of the numbers of gametes that are produced and of the numbers of embryos that are generated in one infusorigen, and we discuss the reproductive capacity of infusorigens.

MATERIALS AND METHODS

Seventeen individual octopuses, Octopus vulgaris, and three cuttlefish, Sepioteuthis lessoniana, were purchased or collected by the authors in the waters off the western coast of Japan. Dicyema orientale from Sepioteuthis lessoniana and three species of dicyemids, namely, D. acuticephalum, D. japonicum, and D. misakiense, from Octopus vulgaris were examined in the present study.

After the host cephalopods had been sacrificed, their renal sacs were taken out and smeared directly on glass slides. Smeared dicyemids were immediately fixed with Carnoy's fixative or with alcoholic Bouin's solution (a mixture of absolute ethanol saturated with picric acid, formalin and acetic acid, 15:5:1, v/v). Specimens fixed with Carnoy's fixative were stained with Feulgen's stain or by the PAS method and were poststained with Ehrlich's hematoxylin and light green. Specimens fixed with alcoholic Bouin's solution were stained with Ehrlich's hematoxylin and light green only. The development of infusorigens in the axial cells of rhombogens was observed with the aid of a light microscope under an oil-immersion objective at a final magnification of 2000 diameters.

RESULTS

Dicyema orientale

(Figs. 1, 2, 7, and Table 1)

At the stage of the transition from the nematogen to the rhombogen, a number of agametes (axoblasts) degenerate. The remaining agametes, that number about 10 to 20, grow larger and undergo an unequal division (Fig. 1a). Each smaller daughter cell loses its cytoplasm and becomes just a nucleus, known as a paranucleus, and it lies near the cell from which it arose (Fig. 1b). The paranucleus stays within the axial cell of the rhombogen and grows to the same size as the axial cell nucleus of the rhombogen. The larger daughter cell, namely, the progenitor of the infusorigen, undergoes a nearly equal division to the two-cell stage (Figs. 2b and c). One of these cells becomes the first oogonium. The other cell undergoes an equal division and produces the first spermatogonium and an axial cell of the infusorigen (Figs. 2c-e). The axial cell of the infusorigen does not divide further, but it increases in size and incorporates the spermatogonium into its cytoplasm (Fig. 2f). The first oogonium remaining on the periphery of the infusorigen divides equally to generate a second oogonium and a primary oocyte (Fig. 2g). In the same way, the second oogonium produces a third oogonium and a primary oocyte. Very early primary oocytes can be distinguished from oogonia since the nucleolus in the former is larger than that in the latter (Figs. 2g and h). In primary oocytes, at the prophase of the first meiotic division, the chromatin becomes aggregated on one side of the

nucleus and the nuclear membrane becomes indistinct on the side opposite the aggregation of chromatin (Figs. 1i and 2l). These features are characteristic of the so-called "bouquet stage" of the prophase of the meiotic division. The primary oocytes gradually become larger and chromosomes become visible as thick threads in the nuclei, features that characterize the zygotene stage. At this stage, both ends of the chromosomes are attached to the nuclear envelope (Figs. 1d and 2j). During the pachytene stage, the chromosomes become indistinct and the nucleus becomes similar in appearance to the interphase nucleus. At this stage, the nucleus includes a very large nucleolus (Figs. 1f and 2k-l). When primary oocytes have grown to about 7 μm in diameter, bead-like chromosomes appear in the nucleus. These oocytes are at the diplotene stage of meiotic prophase (Fig. 1h). The primary oocytes finally reach about 12 μm in diameter (Fig. 1i).

The first spermatogonium within the axial cell of the infusorigen undergoes an equal division and produces a spermatogonium and a primary spermatocyte (Figs. 2h-j). The prophase of the first meiotic division of the primary spermatocyte proceeds similarly to that of the primary oocyte, but the size of the primary spermatocyte does not change throughout the prophase (Figs. 1c, 1d, 2j, and 2k). After the first meiotic division, a pair of secondary spermatocytes enters interkinesis. At this stage, no chromosome structures can be seen. Within the axial cell of maturing infusorigen, usually two secondary spermatocytes are observed in addition to a spermatogonium and an axial cell nucleus. Soon after the second meiotic division, transformation of spermatids into spermatozoa

occurs. Mature spermatozoa are composed of a small amount of deeply stained chromatin and a surrounding small clear area, interpreted as cytoplasm. The cell membrane is hardly visible. The entire spermatozoon is about 2 μm in diameter. The chromatin is usually horseshoe-shaped, but sometimes it is irregularly ring- or dot-shaped (Figs. 1h and 21). After emerging from the axial cell of an infusorigen, the spermatozoon enters the primary oocyte (Fig. 21). Fertilized oocytes remain adhering to the axial cell up to the time at which the first polar bodies are produced. Spermatozoa often adhere to the outer surface of the axial cell, to the oogonia, or to the primary oocytes, or they may appear between the oocyte and the axial cell (Fig. 21). The spermatozoon within the oocyte lies at the periphery of the metaphase plate of the first meiotic division of the oocyte (Fig. 21). The first polar bodies are composed of a mass of chromatin and a clear cytoplasmic area that is surrounded by a delicate membrane. They become detatched from the oocytes and often remain intact, but finally they degenerate.

The numbers of spermatogonia and primary spermatocytes, the number of spermatozoa within and on the surface of the infusorigen, and the numbers of oogonia and primary oocytes per infusorigen are shown in Table 1. D. orientale, being relatively long, has a large number of infusorigens and infusoriform embryos in the axial cell of a rhombogen (Table 1).

Dicyema acuticephalum

(Figs. 3, 4, 7, and Table 1)

One or rarely two agametes become larger and undergo an

unequal division at the beginning of the rhombogen stage (Figs. 3a and 4a). The larger daughter cell is the progenitor of an infusorigen, while the smaller cell becomes a paranucleus (Fig. 3b). The progenitor cell of the infusorigen undergoes an equal division, which results in the two-cell stage (Fig. 4c). One of these cells becomes the first oogonium, and the other cell increases in size and divides unequally. The larger cell, the axial cell of the infusorigen, undergoes no further divisions (Fig. 4d), while the smaller cell divides equally and produces both the first spermatogonium and an oogonium (Fig. 4g). Both cells are so similar in size and appearance that they cannot be distinguished until one of them enters the mitotic phase on the periphery of the infusorigen or is embedded in the axial cell (Fig. 4i). D. acuticephalum has two egg lines (Fig. 7B). The oogonium of each egg line further divides and generates an oogonium and a primary oocyte (Figs. 4e-i). At around the six-cell stage, the first spermatogonium is incorporated into the axial cell of the infusorigen (Fig. 4i). The process of spermatogenesis is similar to that observed in D. orientale. The chromatin of spermatozoa usually forms a horseshoe or an irregular ring (Fig. 4l). The numbers of spermatogonia and primary spermatocytes, and other numerical data, are shown in Table 1.

Dicyema japonicum and Dicyema misakiense

(Figs. 5-7 and Table 1)

The developmental pattern of the infusorigen is the same in D. japonicum and D. misakiense. One or rarely two of the agametes

become larger and undergo an unequal division at the beginning of the rhombogen stage. The smaller cell is transformed into a paranucleus, while the larger cell becomes the progenitor of an infusorigen (Fig. 5a). This latter cell divides unequally and produces two cells (Figs. 5b and 6a). The smaller cell often undergoes an equal division (Fig. 6b), but one of the daughter cells soon degenerates. The remaining cell is the first spermatogonium (Fig. 6c). The larger cell divides equally and, thus, produces the first oogonium and an axial cell of the infusorigen (Figs. 5c and 6d). The axial cell undergoes no further divisions and increases in size. The first spermatogonium is incorporated into the cytoplasm of the axial cell (Fig. 6e) and its nucleus increases in size (Fig. 6f). The oogonium gives rise to a new oogonium and a primary oocyte. Subsequent oogenesis and spermatogenesis proceed in the same manner as in D. orientale and D. acuticephalum. The nucleus of the spermatozoa is horseshoe- or dot-shaped, and the spermatozoa often aggregate (Figs. 5e, 5f, and 6j). The number of spermatozoa and other numerical data are shown in Table 1.

DISCUSSION

In the four species of dicyemid mesozoans studied herein, an agamete enlarged at the beginning of the rhombogen stage. In other species of dicyemids, Nouvel [14] and McConnaughey [12] also reported that an agamete enlarges at the very early stage of the rhombogen. This enlargement is interpreted as a sign of the

beginning of the development of infusorigens. Then the agamete undergoes an unequal division and the smaller daughter cell becomes a paranucleus without contributing to the formation of an infusorigen. Although we can offer no explanation for the formation of a paranucleus, it is a constant feature and may, thus, be essential to the development of infusorigens.

Several differences were apparent in the cell lineage of the infusorigens of the four species (Fig. 7). Nevertheless, two common features were apparent; one is that the first spermatogonium is incorporated into an axial cell, in which spermatogenesis proceeds; and the other is that the oogonium remains at the periphery of the axial cell, where oogenesis occurs. The distinctive differences occur early in the development in the various species. Two patterns of cell lineage are distinguishable. One pattern is characterized by a second division that produces the first oogonium, and the other is characterized by a second division that produces the first spermatogonium. The former is seen in D. orientale and D. acuticephalum, and the latter in D. japonicum and D. misakiense (Fig. 7). In D. orientale, D. japonicum, and D. misakiense, all types of cell differentiate up to the third division. In D. acuticephalum, by contrast, spermatogonia are generated only after the fourth division (Fig. 7B). In spermatogenesis, all four species examined have only one sperm line. In oogenesis, D. acuticephalum has two egg lines, while the other three species have only one (Fig. 7). However, D. acuticephalum has a rather small number of oogonium and oocytes (Table 1). The body size may be a factor that limits the number of oocytes.

The development of infusorigens was reported by Lameere [10], Nouvel [14], and McConnaughey [12]. Lameere studied D. typus, while McConnaughey did not specify the species that he studied. However, both these authors observed that the first oogonium is produced by the second division and the first spermatogonium and axial cell are generated after the third division. This previously reported cell lineage is, thus, the same as that observed in D. orientale. Nouvel [14] traced the development of the infusorigen of D. schulzianum. His findings are identical to ours in D. japonicum and D. misakiense in that the first spermatogonium is produced after the second division and the oogonium and axial cell are produced after the third division. However, the first spermatogonium of D. schulzianum is relatively large compared to that of D. japonicum and D. misakiense. In D. orientale, D. japonicum, and D. misakiense, the first spermatogonia, formed by unequal divisions, are relatively small before they are incorporated into an axial cell. They increase in size until they are as large as oogonia after having been incoporated into an axial cell. In D. acuticephalum, the first spermatogonium is also a small-sized cell. The third division in this species proceeds unequally and generates a larger axial cell and a smaller cell. This smaller cell is the mother cell of both the first spermatogonium and the oogonium of one egg line. Thus, the differentiation of the first spermatogonium occurs later in D. acuticephalum than in the other species (Fig. 7). The pattern of cell lineage observed in D. acuticephalum has not been reported in the earlier literature. Although previous reports dealing with a few species [10, 12, 14] did not pay any attention

to species-specific differences in the cell lineages of infusorigens, a distinct difference does exist and could be a criterion for classification of dicyemid species.

In D. japonicum and D. misakiense, the presumptive first spermatogonium often undergoes equal division before being incorporated into the axial cell. However, one of the daughter cells degenerates soon without differentiating into an oogonium, in contrast to the case in D. acuticephalum. This paticular division might be an "extra" cell division because it does not occur consistently in all infusorigens examined.

In D. aegira, which is about 1.5 mm in total length and has one or two infusorigens, two to three times as many spermatozoa are produced as oocytes [1, 13]. Among the species studied herein, D. orientale is a relatively large dicyemid, reaching 3.5 mm in length, and it has 7 to 25 infusorigens. D. acuticephalum, which is small, and D. japonicum and D. misakiense, which are medium-sized, have usually one, or sometimes two infusorigens [4, 16]. In the four species that we examined, the numbers of spermatozoa and the numbers of the oogonia and primary oocytes were roughly equal (Table 1). The numbers of oogonia and primary oocytes were 2.7 to 3.3 times those of spermatogonia and primary spermatocytes. If spermatogenesis and oogenesis proceed at the same rate during the germ cell division and maturation, the number of spermatozoa can be estimated to be 13 to 16 (3.27 to 4.06 x 4). These values are 1.2 to 1.5 times the values for oocytes. This discrepancy may be attributed to a possibly lower rate of spermatogenesis than of oogenesis [1]. The apparently small number of spermatozoa might

be due to the limited space within the axial cell of the infusorigen. A large number of spermatozoa may not be necessary in dicyemids, which perform self-fertilization within the axial cells of rhombogens. However, it is still unclear why the present four species have much smaller numbers of spermatozoa than D. aegira. It is apparent that the size of rhombogens and the number of infusorigens per rhombogen do not affect the number of gametes produced.

In the nematode Caenorhabditis elegans, the cell lineage of the gonad has been studied in detail [6, 9]. Dicyemids have very simple gonads that are composed of a very small number of cells, and thus, they may also prove to useful as model systems for studies of the differentiation of gametes.

SUMMARY

The development of the functionally hermaphroditic gonad, the infusorigen, in four dicyemid species, Dicyema orientale, D. acuticephalum, D. japonicum, and D. misakiense, was studied in fixed materials with the aid of a light microscope. After an agamete (axoblast) undergoes the first division and excludes a paranucleus, the resulting cell undergoes the second division. Afterwards, three different types of cell lineage can be identified. (1) In D. orientale, the first oogonium is produced by the second division, and the axial cell of an infusorigen and the first spermatogonium are produced by the third division. (2) In D. acuticephalum, the first oogonium is produced by the

second division, the axial cell is produced by the third division, and the spermatogonium is produced by the fourth division. The fourth division also produces the first oogonium of another egg line. (3) In D. japonicum and D. misakiense, the first spermatogonium is produced by the second division, and the axial cell and the first oogonium are produced by the third division. In all species examined, oogonia occupy the outer surface of the axial cell and spermatogonia are incorporated into the axial cell. In this way, the spermatogenesis proceeds within the cytoplasm of the axial cell. Mature infusorigens of these four species consist of about twenty cells. The respective numbers of oocytes and spermatozoa produced in each infusorigen are roughly equal in these four species.

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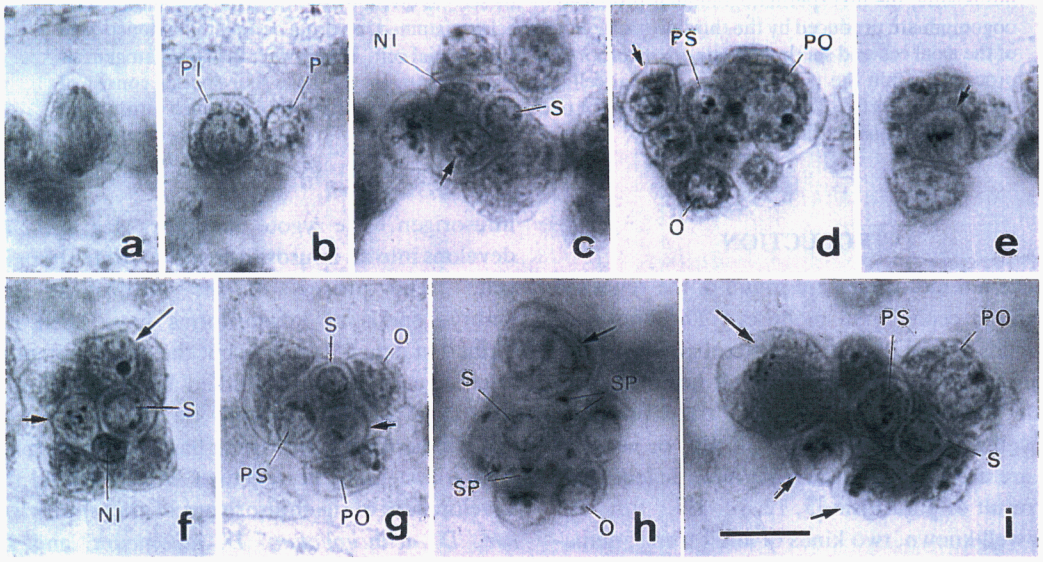

Fig. 1. Light micrograghs of developing infusorigens within the axial cells of rhombogens of D. orientale. Photographs were taken at a magnification of 2000 diameters under an oil-immersion objective. Scale bar represents 10 μm. (a): An agamete undergoing an unequal first division. (b): A progenitor cell of an infusorigen (PI) and a paranucleus (P) being produced after the first division. (c)-(i): Infusorigens. (c): The arrow indicates the zygotene stage of a primary spermatocyte. (d): The arrow indicates the zygotene stage of a primary oocyte. (e): The arrow indicates the metaphase of a primary spermatocyte. (f): The short arrow indicates the metaphase of a secondary spermatocyte viewed from the pole and the long arrow indicates the pachytene stage of a primary oocyte. The nucleolus is conspicuosly large in the primary oocyte at the pachytene stage. (g): The arrow indicates the metaphase of a secondary spermatocyte viewed from the side. (h): The arrow indicates the diplotene stage of a primary oocyte. (i): The short arrows indicate the bouquet stage of a primary oocyte and the long arrow indicates the anaphase of a primary oocyte.

NI, Axial cell nucleus of infusorigen; O, oogonium; PO, primary oocyte; PS, primary spermatocyte; S, spermatogonium; SP, spermatozoon.

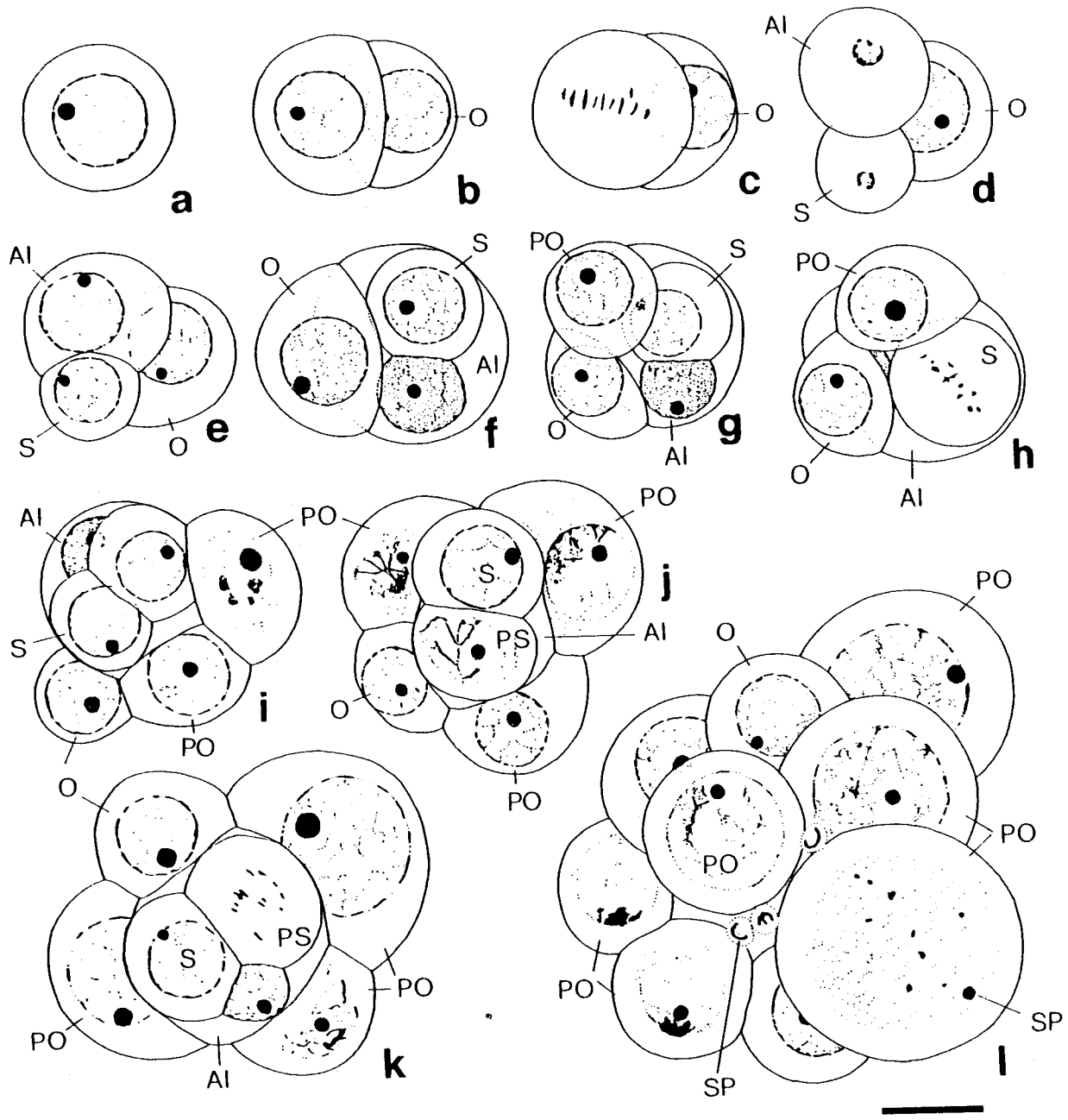
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Fig. 2

Fig. 2. Sketches of the development of the infusorigen of D. orientale. Bar represents 5 μm. (a): A progenitor cell of infusorigen. (b) and (c): Two-cell stage. In (c), the metaphase of the third division is seen. (d)-(f): Three-cell stage. In (d) and (e), both the axial cell (AI) and the first spermatogonium (S) produced after the third division are shown. In (f), the first spermatogonium (S)is embedded in the axial cell (AI). (g): Four-cell stage. (h): Five-cell stage. The metaphase of the first spermatogonium (S)is seen. (i): Six-cell stage in optical section. (j): Eight-cell stage in optical section. Primary oocyte (PO) at the upper right corner is in the zygotene stage. (k): Nine-cell stage in optical section. The anaphase of the primary spermatocyte (PS) is seen. (l): The infusorigen. A primary oocyte (PO) at the beginning of anaphase is seen in the lower right corner. This oocyte contains a spermatozoon (SP) in the cytoplasm. Spermatozoa (SP) in the center are emerging from the axial cell of the infusorigen.

AI, axial cell of infusorigen; O, oogonium; PO, primary oocyte; PS, primary spermatocyte; S, spermatogonium; SP, spermatozoon.

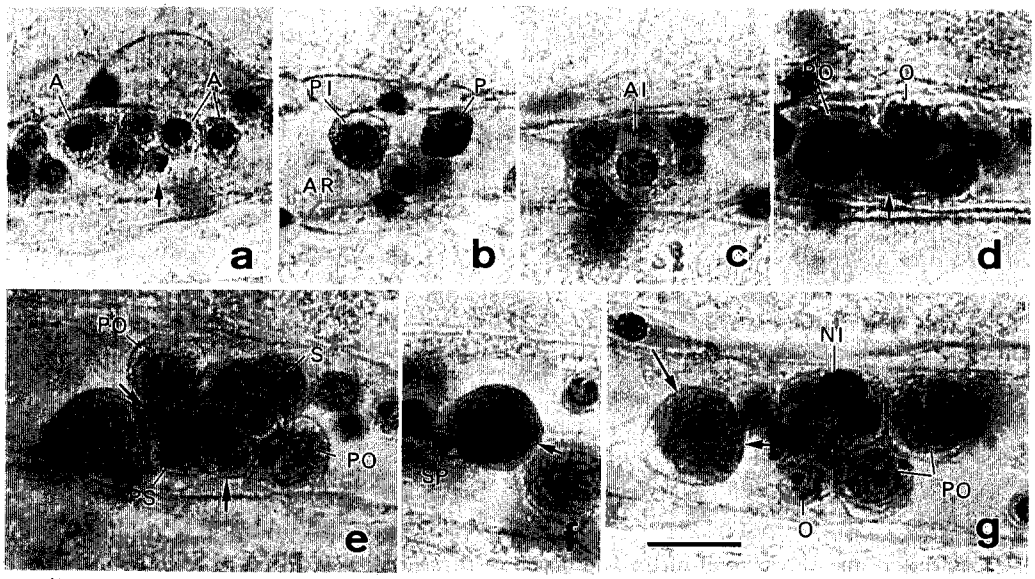

Fig. 3. Light micrographs of developing infusorigens within the axial cells of rhombogens of D. acuticephalum. Bar represents 10 um. (a): Agametes (A) A telophase figure of the first unequal division is seen in the center. The arrow indicates a smaller daughter cell that becomes a paranucleus. (b): A progenitor cell of an infusorigen (PI), and a paranucleus (P) within an axial cell (AR) of a rhombogen. (c): Five-cell stage. (d)-(g): Infusorigens. (d): The arrow indicates the metaphase of a primary spermatocyte viewed from the side. (e): The arrows indicate the interkinesis stage of the secondary spermatocytes. (f): The metaphase of a primary oocyte viewed from the side. The arrow indicates a sperm within the oocyte. (g): The large arrow indicates the anaphase of a secondary oocyte. The short arrow indicates a sperm within it. The primary oocyte (PO) is at the pachytene phase.

AI, axial cell of infusorigen; NI, axial cell nucleus of infusorigen; O, oogonium; PO, primary oocyte; PS, primary spermatocyte; S, spermatogonium; SP, spermatozoon.

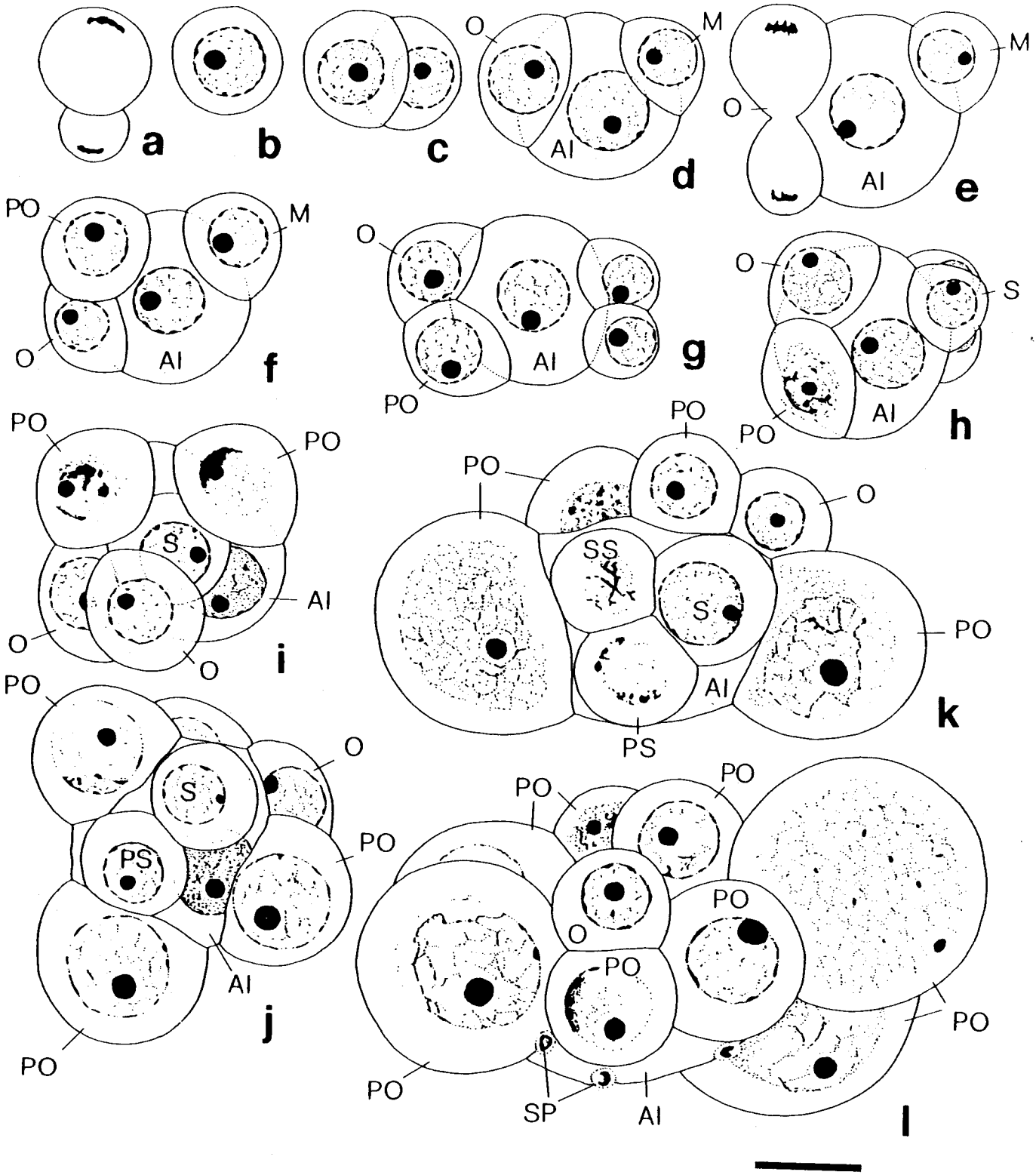
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Fig. 4

Fig. 4. Sketches of the development of the infusorigen of D. acuticephalum. Bar represents 5 μm. (a): The telophase of the first division of the agamete. The smaller cell becomes a paranucleus. (b): The progenitor cell of an infusorigen. (c): Two-cell stage. (d)-(e): Three-cell stage. In (e), the telophase of an oogonium (O) is seen. The cell marked M is a mother cell of an oogonium and a spermatogonium. (f): Four-cell stage. (g): Five-cell stage. Two cells on the right side of the axial cell, produced by the division of the mother cell (M in e and f), become the first spermatogonium and the oogonium of one egg line, respectively. (h)-(i): Six-cell stage. In (i), the first spermatogonium (S) is embedded in the axial cell (AI) and the primary oocytes (PO) are at the bouquet stage. (j): Eight-cell stage in optical section. (k): An infusorigen in optical section. A secondary spermatocyte (SS) is seen in the axial cell (AI). (l): An infusorigen (surface view). The anaphase of a primary oocyte (PO)is seen in the upper right corner.

AI, axial cell of infusorigen; M, mother cell of the first spermatogonium and the oogonium; O, oogonium, PO, primary oocyte; PS, primary spermatocyte; S, spermatogonium; SP, spermatozoon; SS, secondary spermatocyte.

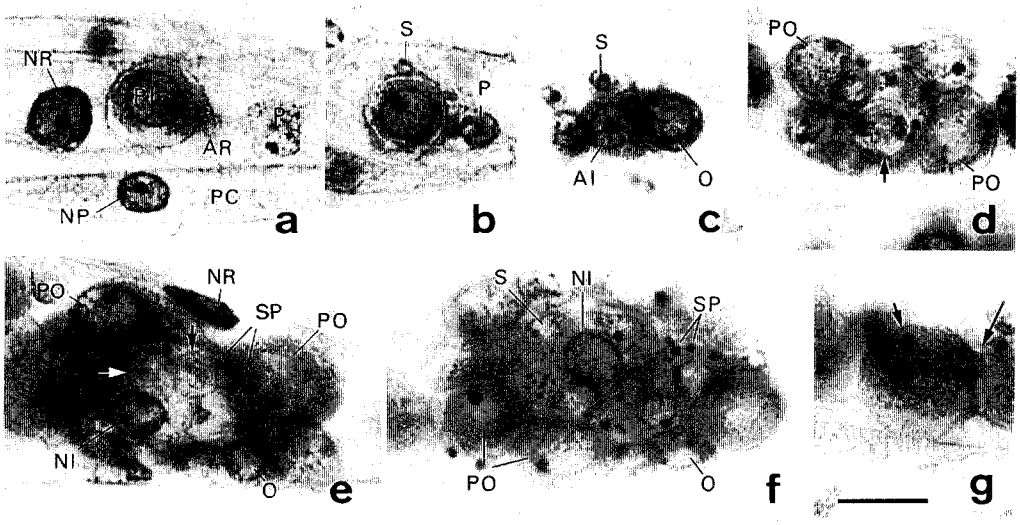

Fig. 5. Light micrographs of developing infusorigens within the axial cells of rhombogens of D. japonicum. Bar represents 10 μm. (a): A progenitor cell of an infusorigen (PI), the axial cell of a rhombogen (AR), a peripheral cell nucleus (NP), an axial cell nucleus of a rhombogen (NR), a paranucleus (P), and peripheral cell cytoplasm (PC) can be seen. (b): Two-cell stage. (c): Three-cell stage. (d)-(f): Infusorigens. (d): The arrow indicates the metaphase of the primary spermatocyte. (e): The arrows indicate the interkinesis stage of a secondary spermatocyte. (f): The arrow indicates the metaphase of a secondary spermatocyte viewed from the pole. (g): The telophase of a primary oocyte. The long arrow indicates the first polar body. The short arrow indicates the sperm within the primary oocyte.

AI, axial cell of infusorigen; NI, axial cell nucleus of infusorigen; NR, axial cell nucleus of rhombogen; O, oogonium; P, paranucleus; PO, promary oocyte; S, spermatogonium; SP, spermatozoon.

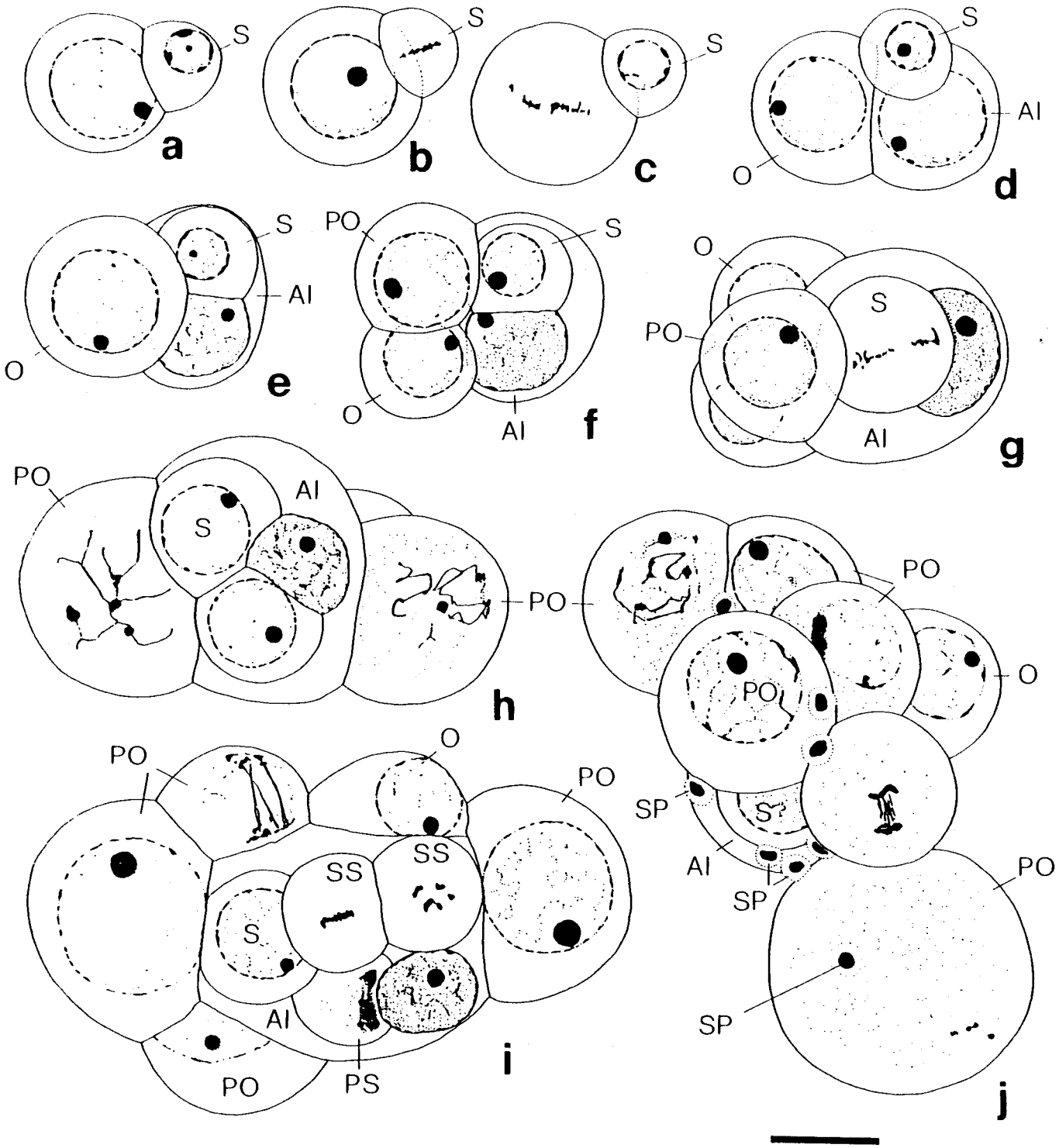
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Fig. 6

Fig. 6. Sketches of the development of infusorigens of D. misakiense. Bar represents 5 μm. (a)-(c): Two-cell stage. As seen in (b), the first spermatogonium (S) often divides quite early. In (c), the mother cell of both the first oogonium and the axial cell is in metaphase. (d)-(e): Three-cell stage. In (e), a spermatogonium (S) is embedded in the axial cell (AI). (f): Four-cell stage. (g): Five-cell stage. The metaphase of a spermatogonium (S) is seen. (h): Eight-cell stage in optical section. The primary oocytes (PO) at the zygotene stage are seen. (i): The infusorigen in optical section. The primary oocytes (PO at the left and right) at the pachytene stage and another oocyte (PO at the upper left corner) at the zygotene stage are seen. The metaphases of the secondary spermatocytes (SS) are also seen from the side (center) and from the pole (right). (j): The infusorigen (surface view). Some spermatozoa (SP) are emerging from the axial cell.

AI, axial cell of infusorigen; O,oogonium; PO, primary oocyte; PS, primary spermatocyte; S, spermatogonium; SP, spermatozoon; SS, secondary spermatocyte.

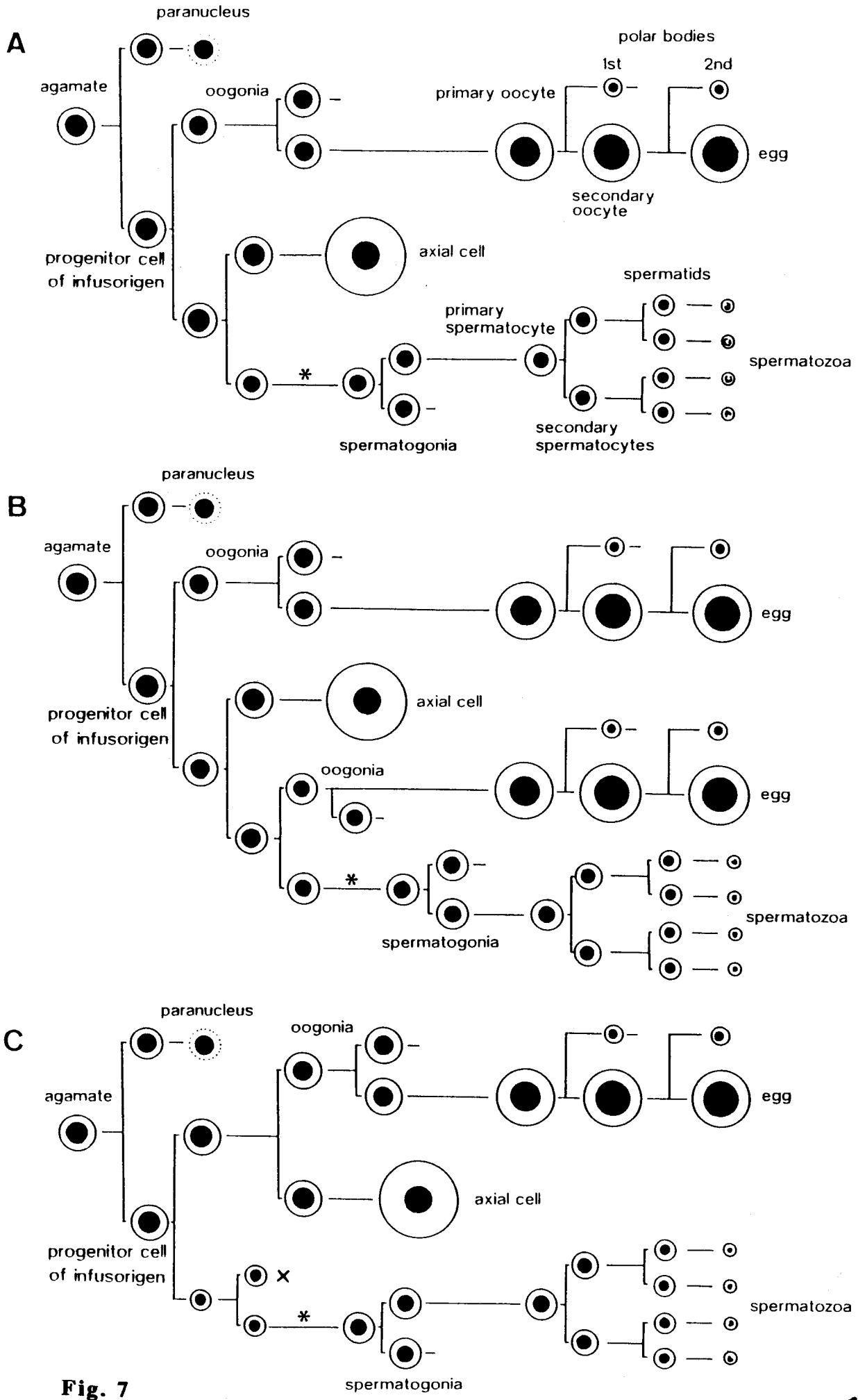
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Fig. 7

Fig. 7. The cell lineage of an infusorigen. (A): D. orientale. (B): D. acuticephalum. (C): D. japonicum and D. misakiense. The circle represents the cell and the central black area represents the nucleus. They are nearly scaled. The dotted circle means that the cytoplasm degenerates. At the site of the asterisk (*), the first spermatogonium is embedded in the axial cell. Short horizontal lines on right side of cells indicate that the cells proliferate further. In (c), the cross (x) means that the cell does not proliferate but degenerates.

Table 1. The numbers of infusorigens, gametes, and embryos within the axial cells of rhombogens

Species	Body length of rhombogens (mm)	No. of infusorigens per rhombogen	No. of spermatogonia and primary spermatocytes per infusorigen[1)]
Dicyema orientale[2)]	~3.5	7–25	3.27±0.71
D. acuticephalum	~0.8	1– 2	3.38±0.86
D. japonicum	~1.0	1– 2	4.06±1.28
D. misakiense	~1.0	1– 2	4.04±1.24

Species	No. of spermatozoa per infusorigen[1)]	No. of oogonia and primary oocytes per infusorigen[1)]	No. of infusorform embryos per rhombogen
D. orientale[2)]	11.78±5.21	10.26±2.30	~250
D. acuticephalum	12.67±4.70	9.25±1.43	~ 20
D. japonicum	10.00±3.91	13.43±3.47	~ 35
D. misakiense	11.59±4.70	12.04±3.76	~ 35

1) Values represent means ± S.D. and are based on results from 50 mature infusorigens.

2) D. orientale was described only with nematogens [16] and no rhombogens have been reported.

CHAPTER IV

The Development of the Vermiform Embryos of Two Mesozoans, *Dicyema acuticephalum* and *Dicyema japonicum*

INTRODUCTION

Dicyemid mesozoans are found in the renal sac of benthic cephalopod molluscs. The bodies of dicyemids consist of only 20 to 40 cells and are organized in a very simple fashion [14, 15]. It has long been debated whether dicyemids are truly primitive multicellular animals [2, 10, 11, 13, 17], or they are actually organisms that have degenerated as a result of parasitism [7, 16, 18].

Two adult forms of dicyemids, namely, nematogens and rhombogens, are found. Asexual reproduction occurs within the axial cells of nematogens and vermiform embryos develop from agametes (axoblasts), while sexual reproduction takes place within the axial cells of rhombogens. A hermaphroditic gonad, which is called an infusorigen, is formed within the axial cell and fertilization occurs around the infusorigen. The zygote undergoes cleavages and develops into an infusoriform embryo within the axial cell. The processes of gametogenesis and cleavage have recently been described in detail [4, 5]. The development of vermiform embryos was described in the early literature [6, 10 cited in 14, 12, 14, 16], but the pattern of cell divisions and the process of cell arrangement during embryogenesis remain to be established. Moreover, cell lineages have not been completely characterized. In this report, we describe details of the development of the vermiform embryo of Dicyema acuticephalum, which has from 16 to 18 peripheral cells [16], and of Dicyema japonicum, which has 22 peripheral cells

[3]. Dicyemids are examples of animals with a fixed cell number and their somatic cells undergo only a limited number of divisions during embryogenesis. The analysis of embryonic cell lineages in dicyemids is important since it provides clues towards an understanding of a simple or basic pattern of cell differentiation in multicellular animals.

MATERIALS AND METHODS

Forty-seven octopuses, Octopus vulgaris, were purchased or collected by the authors in the waters off the western coast of Japan. In this region, four species of dicyemids are found in the kidneys of Octopus vulgaris [3]. In this study, only Dicyema acuticephalum and Dicyema japonicum were examined.

After sacrifice, the renal sacs of each were remove and smeared directly on glass slides. Smeared dicyemids were immediately fixed with Carnoy's fixative or with alcoholic Bouin's solution (a mixture of absolute ethanol saturated with picric acid, formalin and acetic acid, 15:5:1, v/v). Specimens fixed with Carnoy's fixative were stained with Feulgen's stain or by the PAS method and were poststained with Ehrlich's hematoxylin and light green. Specimens fixed with alcoholic Bouin's solution were stained with Ehrlich's hematoxylin and light green only. The embryos in the axial cells of nematogens were observed under a light microscope with an oil-immersion objective at a final magnification of 2,000 diameters. Cells were identified by various criteria, such as the position within the embryo, the

size of the nucleus and the cell, and the stainability of the nucleus and the cell. Paying careful attention, we identified each swollen nucleus that was about to divide and each metaphase figure in terms of the cell that was going to divide and the resultant two daughter cells. Each developing embryo with or without dividing cells was sketched at three different optical depths and a three-dimensional diagrams were generated from these sketches.

The early division of the vermiform embryos is somewhat spiral, but not absolutely so, and it proceeds cell by cell and not by quartets. Therefore, a new terminology was developed to describe the cells. Although the body of a vermiform specimen does not differentiate into a dorsal and a ventral side, or a left and a right side, the embryos are apparently formed bilaterally during embryogenesis. In order to facilitate descriptions, a dorso-ventral axis for the embryo was tentatively defined. The cells of the vermiform were named in accordance with the nomenclature of earlier authors [14, 16].

Terminology for identification of cells

At the two-cell stage, the two cells are designated A and B. Cell A divides and produces two daughter cells. One is designated 2a, the prospective axial cell, while the other is designated as 2A, the mother cell of peripheral cells. The first digit is equal to the cell generation, namely, the number of prior cell divisions. At the four-cell stage, two daughter cells

of cell B are situated on the tentatively defined left and right sides of the embryo. The cell on the right side is distinguished from the cell on the left by underlining. Thus, the left and right cells are designated as 2B and <u>2B</u>, respectively. Cell 2B produces two daughter cells. The anterior cell is designated $3B^1$ and the posterior one is designated $3B^2$. Thus, the anterior and posterior daughter cells of $3B^1$ are designated $3B^{11}$ and $3B^{12}$, respectively.

RESULTS

At the nematogen stage, an agamete divides equally and produces two separate daughter agametes. They increase in number by mitosis, and some of develop asexually into vermiform embryos within the axial cell of the nematogen (Figs. 1, 2a, and 8a).

<u>Dicyema</u> <u>acuticephalum</u>; the type with 16 peripheral cells (Figs. 2, 3, 4, and 5)

Before the first division, an agamete occasionally undergoes an extremely unequal division (Fig. 3a). The resultant much smaller cell remains attached to the larger one but it ultimately degenerates without contributing to embryogenesis. The first division is meridional and equal, producing two daughter cells, A and B (Fig. 3b). Cell B is the mother cell of the head peripheral cells. The second division involves only cell A. This division is latitudinal and equal, producing two daughter

cells, 2A and 2a (Figs. 3c and d). Cell 2A is the mother cell of the peripheral cells of the trunk and tail, while cell 2a is the prospective axial cell. The third division involves cell B. This division is meridional and equal, producing two daughter cells, 2B and 2B (Fig. 3e). At this four-cell stage, two pairs of cells, 2A-2a and 2B-2B, are arranged crosswise with respect to one another. The third division furrow coincides with the plane of bilateral symmetry of the embryo. The pattern of division and the cell lineage of descendants of cell 2B are the same as those of cell 2B.

The fourth division involves at cell 2A. This division is also meridional and equal, resulting in the five-cell stage (Figs. 3f-h). The division plane again coincides with the plane of bilateral symmetry and it separates the left cell (3A) from the right cell (3A). The division pattern and the cell lineage of descendants of cell 3A are the same as those of cell 3A. At around the five-cell stage, cell 2a, the prospective axial cell, undergoes an extremely unequal division and produces two daughter cells that are quite different in size (Figs. 3i and j). The larger cell, 3a, retains the characteristic of the parent cell, while the much smaller cell degenerates and ultimately disappears during embryogenesis. Cell 3a gradually becomes larger prior to the next division.

The pattern of cell division beyond the five-cell stage changes from spiral to bilateral. After the five-cell stage, divisions occur not one by one but in pairs, and they become almost synchronous. Therefore, subsequent developmental stages proceed with odd numbers of cells, yielding, for example, a

seven-cell stage, and so on. The fifth division is an equal division and results in the seven-cell embryo (Figs. 3k and l). Thus, cells 2B and $\underline{2B}$ divide and produce two pairs of daughter cells, $3B^1$ and $3B^2$ plus $\underline{3B^1}$ and $\underline{3B^2}$, respectively. The future anterior-posterior axis of the embryo corresponds almost exactly to the $3B^1$-3A axis of the seven-cell embryo. The sixth division is extremely unequal (Fig. 3m). Cells $3B^1$ and $\underline{3B^1}$ divide and together they produce a pair of large cells and a pair of much smaller daughter cells. Although they remain in place on the developing embryo until later stages, the smaller cells eventually degenerate and disappear. At this stage, cell 3a again divides unequally into a larger daughter cell, 4a, and a much smaller daughter cell which degenerates and ultimately disappears (Figs. 3n and o).

The seventh division is slightly unequal. Cells 3A and $\underline{3A}$ divide into two pairs of daughter cells, $4A^1$ and $4A^2$ plus $\underline{4A^1}$ and $\underline{4A^2}$ (Figs. 3o, p). Cells $4A^2$ and $\underline{4A^2}$ are the smallest cells at this stage. They do not divide further but become the diapolar cells. At the nine-cell stage, the $3B^2$ pair undergo extremely unequal divisions, to form the larger cells $4B^2$ and $\underline{4B^2}$ and two much smaller cells (Fig. 3q). The much smaller cells remain around the larger cells until later stages (Fig. 2c) but they degenerate and ultimately disappear, while the larger cells undergo no further divisions and become the parapolar cells. The $4A^1$ pair divide equally and produce two pairs of daughter cells, $5A^{11}$ and $5A^{12}$ plus $\underline{5A^{11}}$ and $\underline{5a^{12}}$ (Figs. 3r and s). Neither pair divides further and these cells become diapolar cells and uropolar cells. At around the nine-cell stage, cell 4a again

undergoes an extremely unequal division (Fig. 3p). The resultant much smaller cell remains between the axial cell and the peripheral cells until later stages, but it finally disappears (Fig. 2d). As peripheral cells are formed, the larger daughter cell, 5a, is gradually enveloped by peripheral cells. Soon, the prospective axial cell, 5a, is completely surrounded by peripheral cells (Fig. 3v). Then cell 5a again divides unequally into a larger daughter cell, 6a, and a much smaller daughter cell. The smaller cell remains for a while between the prospective axial cell and the peripheral cells but it ultimately disappears during embryogenesis.

The thirteen-cell stage is achieved by equal divisions of cells $4B^1$ and $\underline{4B^1}$ (Figs. 3t and u). The resultant cells $5B^{11}$ and $5B^{12}$ divide again into two pairs of daughter cells, $6B^{111}$ and $6B^{112}$ plus $6B^{121}$ and $6B^{122}$, in the anterior part of the embryo (Figs. 4a-e). These cells undergo no further divisions, and the $6B^{111}$ and $6B^{121}$ pair become the propolar cells, while the $6B^{112}$ and $6B^{122}$ pair become the metapolar cells. The lineage of cell $\underline{4B^1}$ is the same as that of cell $4B^1$. At the same time, the internal cell 6a, namely the prospective axial cell, divides equally into two daughter cells. The anterior cell, $7a^1$, becomes an axial cell and the posterior one, $7a^2$, becomes the first agamete (Figs. 2e and 4b). The agamete is soon incorporated into the axial cell (Figs. 2e and f). A pair of parapolar cells situated in the dorsal region elongate and approach each other in the ventral region (Fig. 4f). Then, the peripheral cells become ciliated. Cilia on the propolar and metapolar cells are more densely distributed than those on the other peripheral cells.

The fully formed embryo consists of sixteen peripheral cells and one axial cell, which contains two to four agametes (Figs. 2h and 4f). Further development involves only the enlargement and intracellular differentiation of cells that have already formed (Fig. 2f-h). The body length, excluding cilia, of the fully formed embryo is about 55 μm and the body width is about 11 μm.

Dicyema acuticephalum; the type with 17 or 18 peripheral cells (Figs. 6 and 7)

At the thirteen-cell stage of the embryo, which ultimately has 18 peripheral cells, the $4B^2$ pair divide equally to produce two pairs of daughter cells, the $5B^{21}$ and $5B^{22}$ pairs (Figs. 6a and b). The anterior $5B^{21}$ pair become parapolar cells, while the posterior $5B^{22}$ pair become the fourth diapolar cells (Fig. 6c). In the embryo that finally has 17 peripheral cells, terminal division of neither cell $4B^2$ nor cell $\underline{4B^2}$ occurs. Other aspects of embryogenesis are the same as those described above for D. acuticephalum with 16 peripheral cells.

Dicyema japonicum (Figs. 8, 9, 10, and 11)

Up to the nine-cell stage, the pattern of development and the cell lineage in D. japonicum are the same as those described for D. acuticephalum. In the 2a line, extremely unequal divisions occur at around the five-, seven-, nine-, and seventeen-cell stages (Figs. 8c, d and 11).

At the eleven-cell stage, in D. japonicum, the $3B^2$ pair of cells divide equally into $4B^{21}$ and $4B^{22}$ pairs (Figs. 9a-c). Almost simultaneously, the cells of the $3B^1$ pair undergo extremely unequal divisions, generating the larger daughter pair $4B^1$ and $\underline{4B^1}$ and a much smaller pair (Figs. 9d and e). The smaller pair of cells degenerate and finally disappear. At the thirteen-cell stage, the $5A^{11}$ pair divide equally and produce two pairs of daughter cells, $6A^{111}$ and $6A^{112}$ plus $\underline{6A^{111}}$ and $\underline{6A^{112}}$ (Fig. 9f). The plane of this division is parallel to the antero-posterior axis in contrast to the previous division that occurs parallel to the dorso-ventral axis. As the result, cells $6A^{111}$ and $\underline{6A^{111}}$ are situated on the left and right sides of the embryo, respectively.

The $4B^{22}$ pair divide equally and produce two pairs of daughter cells, $5B^{221}$ and $5B^{222}$ plus $\underline{5B^{221}}$ and $\underline{5B^{222}}$ (Fig. 9g). Cells $5B^{221}$ and $\underline{5B^{221}}$ and cells $5B^{222}$ and $\underline{5B^{222}}$ undergo no further divisions and become parapolar cells and diapolar cells, respectively (Figs. 10a and c).

At the seventeen-cell stage, the $5A^{12}$ pair undergo slightly unequal divisions and produce two pairs of daughter cells, $6A^{121}$ and $6A^{122}$ plus $\underline{6A^{121}}$ and $\underline{6A^{122}}$ (Fig. 9h). Neither pair divide further. Cells $6A^{121}$ and $\underline{6A^{121}}$ become uropolar cells, while cells $6A^{122}$ and $\underline{6A^{122}}$ become diapolar cells (Figs. 10a and c).

The $4B^1$ pair divide equally into two pairs of daughter cells, $5B^{11}$ and $5B^{12}$ plus $\underline{5B^{11}}$ and $\underline{5B^{12}}$ (Figs. 9i and j). Soon after these divisions, the $4B^{21}$ pair divide equally into two pairs of daughter cells, $5B^{211}$ and $5B^{212}$ plus $\underline{5B^{211}}$ and $\underline{5B^{212}}$ (Figs. 9k and l). $5B^{211}$ and $\underline{5B^{211}}$ become propolar cells, while cells

$5B^{212}$ and $\underline{5B^{212}}$ become metapolar cells. At around this stage, the prospective axial cell, 6a, divides unequally (Figs. 8e and f). The anterior large cell, $7a^1$, undergoes no further divisions and becomes an axial cell, while the posterior small cell, $7a^2$, becomes an agamete and is soon incorporated into the axial cell (Figs. 8g, h, and 10b). A pair of parapolar cells, situated in the dorsal region, elongate and approach each other in the ventral region (Figs. 10a and c). The vermiform embryo finally consists of twenty-two peripheral cells and one axial cell, which contains one or two agametes (Fig. 8i). The body length, excluding cilia, of the fully formed embryo is about 65 μm and the body width is about 12 μm. No variations in cell lineage were found among embryos examined.

DISCUSSION

The patterns of development of the vermiform embryos of Dicyema acuticephalum and Dicyema japonicum, as described in detail, are very similiar. However, these patterns are very different from those described for Microcyema vespa and Pseudicyema truncatum by Lameere [12], for Dicyema typus by Gersch [6], and for Dicyema balamuthi, Dicyemennea abelis, and Dicyemennea californica by McConnaughey [14].

In both species studied here, the first division is equal and produces two daughter cells of equal size. However, in other dicyemid species, namely, Microcyema vespa, Pseudicyema truncatum [12], Dicyema typus [6], Dicyema balamuthi, Dicyemennea abelis

and Dicyemennea californica [14], the first division is unequal and the two daughter cells are of different sizes. In this study, we noted that one of the two equal daughter cells enlarges after the division, as observed by Hartmann [8 cited in 14]. There may be at least two patterns that typify the first cell division in the various species of dicyemid. In earlier descriptions [6, 12, 13, 14, 16], one of the daughter cells (usually the larger one) is reported to become a prospective axial cell and the other is regarded as the mother cell of the peripheral cells. However, the prospective axial cell is not generated prior to the four-cell stage in D. acuticephalum and D. japonicum. This type of species-specific difference during embryogenesis is reminiscent of that observed in the case of the development of infusorigens [5]. In addition, species difference between D. acuticephalum and D. japonicum was found in the lineage of the cells that form the calotte (Figs. 5 and 11). Research into the cell lineage of vermiforms may be of relevance to the taxonomy of dicyemids.

As shown clearly in Figures 5 and 7, the difference in terms of the peripheral cell number among individual specimens of D. acuticephalum is due to the number of divisions of the $4B^2$ pair. In a previous paper [3], we reported that the peripheral cell number of D. acuticephalum was consistently 18. In the subsequent study of individuals from a large number of octopuses, we noticed, however, specimens of D. acuticephalum with 16 or 17 peripheral cells. In the original description of this species, Nouvel [16] also reported that the peripheral cell number of D. acuticephalum is usually 18 but occasionally 16. In other

dicyemid species such as Dicyema bilobum and Dicyema benthoctopi, which have variable numbers of peripheral cells [1, 9], additional terminal divisions may occur towards the end of the establishment of a certain cell lineage, as in the case of the $4B^2$ pair in D. acuticephalum. The species-specific difference in the peripheral cell number between D. acuticephalum and D. japonicum can be attributed to the number of divisions of the $4A^1$ pair (Figs. 5 and 11). Thus, the $4A^1$ pair in D. japonicum apparently have a greater ability to divide than the $4A^1$ pair of D. acuticephalum. By contrast, the $4A^2$ pair cease divisions early in embryogenesis and soon become the peripheral cells in both species. The number of mother-cell divisions clearly plays a significant role in the morphogenesis of vermiforms. In dicyemids that have relatively large numbers of peripheral cells, mother cells, such as the $4A^1$ pair, may undergo further divisions until the species-specific number of cells has been reached.

The elimination of chromatin during early embryogenesis from the prospective axial cell has been described in some dicyemid species [14]. In D. acuticephalum and D. japonicum at least, we observed not the elimination of chromatin but extremely unequal cell divisions that resulted in the pycnotic degeneration of the smaller daughter cells. Some earlier workers also reported extremely unequal divisions in the case of the prospective axial cell, but they did not report such divisions do not occur in any of peripheral cell lines [12, 16]. By contrast, McConnaughey described the occurrence of extremely unequal divisions in the peripheral cell line [14, 15]. In D. acuticephalum and D. japonicum, extremely unequal division occurs not only in the case

of the prospective axial cell (2a-line), but also at the one-cell stage (agamete) and in the case of the mother cells of the peripheral cells, namely, cells $3B^1$ and $3B^2$ plus $\underline{3B^1}$ and $\underline{3B^2}$ (in D. acuticephalum) or cells $3B^1$ and $\underline{3B^1}$ (in D. japonicum). These phenomena may be examples of programmed cell death. The production of these smaller cells, which are destined to die, appears to be a constant feature found in the embryogenesis of vermiforms.

In D. acuticephalum and D. japonicum, the prospective axial cell (2a-line) divides four times and produces four smaller cells anteriorly until the first agamete is produced posteriorly. In his description of the development of vermiforms of Microcyema vespa and Pseudicyema truncatum, Lameere [12] noted that one small cell is formed by the unequal division of the prospective axial cell and that this small cell itself divides once or twice to produce two to four smaller cells. However, no divisions of the smaller cell itself could be observed in D. acuticephalum and D. japonicum.

Hartmann [8 cited in 14] considered that the number of the much smaller cells was consistent with the number of extra axial cells in the stem nematogen. However, the actual number of these smaller cells that we observed was more than he postulated. The number of these smaller cells was larger in our specimens than the number (usually three) of axial cells in the stem nematogen. It is difficult to provide a reasonable interpretation of this phenomenon. McConnaughey [15] maintained that the formation of the smaller cells represents a relict condition of ancestral forms that had more internal cells and that these small cells

have no particular function in the present species. If an extremely unequal division of a prospective axial cell represents a phenomenon that is derived secondarily from an equal division during what has been, most likely, a long history of parasitism, smaller cells that are destined to die might be expected to be formed ultimately. The vermiforms might originally have had some internal cells, as McConnaughey suggested. The prospective axial cell has at least the ability to divide further and produce some internal cells, even though these cells are actually fated to die. It seems possible that these successive, extremely unequal divisions in the 3a-line might contribute to the maintenance of increased amounts of cytoplasm in the resultant larger cell. The larger cell retains most of the cytoplasm of the mother cell and enlarges after each division. The prospective axial cell may require a large amount of cytoplasm to accommodate a prospective agamete.

We must also consider the fact that extremely unequal divisions occur consistently in the cell lineage of the 3B-line and not in 3A-line. The 3B-line gives rise to the head region, which includes the calotte, which has distinctive features among peripheral cells. By contrast, the 2A-line gives rise to the trunk and tail region which are composed of standard peripheral cells. It is likely that extremely unequal divisions in the peripheral cell lineage are somehow associated with the characteristic differentiation of cells. The prospective axial cell also has distinct features and undergoes extremely unequal divisions.

Programmed cell death has also been reported in the

development of infusoriforms [4]. Moreover, McConnaughey [14] and Nouvel [16] found that embryos of stem nematogens include a number of degenerating cells. Thus, the formation of much smaller cells that are destined to die during embryogenesis appears to be a constant and general feature of development of dicyemids. In the embryogenesis of infusoriform embryos, a cell line that includes a programme for cell death gives rise to remarkably differentiated cells, such as the capsule cell [4]. Although we can offer no reasonable explanation at present, the programmed cell death might somehow be involved in an accelation of cell differentiation.

In the embryogenesis of vermiforms, cell divisions are determinate and result in an embryo with a definite number and arrangement of cells. The developmental process of the vermiform embryo seems very simple and it seems to be programmed similarly to that of the infusorigen and the infusoriform embryo [4, 5]. There appears to be little plasticity in such development because of the simple body organization of these organisms.

The constant numbers of cells in dicyemids is strongly reminiscent of that of aschelminths. McConnaughey [15] suggested that dicyemids may possibly be related to very early progenitors of aschelminths or to certain of the earliest aschelminths. Programmed cell death has also been noted in the embryogenesis of the nematode Caenorhabditis elegans [19]. However, there is apparently no further evidence to support any relationship between dicyemids and aschelminths.

SUMMARY

The pattern of cell division and the cell lineage of the vermiform embryos of dicyemid mesozoans were studied under the light microscope using fixed and stained specimens of two species, namely, Dicyema acuticephalum, which has 16 to 18 peripheral cells, and Dicyema japonicum, which has 22 peripheral cells. An agamete first divides into two apparently equivalent daughter cells which remain in contact with one another. One of these cells becomes the mother cell of the head of the embryo. The other cell divides again equally to produce the prospective axial cell and the mother cell of the trunk and the tail of the embryo. The division proceeds spirally in the early stages but becomes bilateral from the fifth cell division onward. The embryo finally exhibits apparently bilateral symmetry. In two lines of cells, namely, those descended from the prospective axial cell and those from the mother cell of the head, extremely unequal divisions occur and the resultant, much smaller cells from each unequal division degenerate and ultimately disappear during embryogenesis. At the thirteen-cell stage, peripheral cells surround the prospective axial cell. At the final stage of embryogenesis, the prospective axial cell divides into two daughter cells. The anterior one is the axial cell itself and the posterior one is incorporated into the axial cell to form an agamete. Differences in numbers of peripheral cell are due to the number of times that divisions of the mother cells occur. The cell lineage of the calotte differs between D. acuticephalum and D. japonicum.

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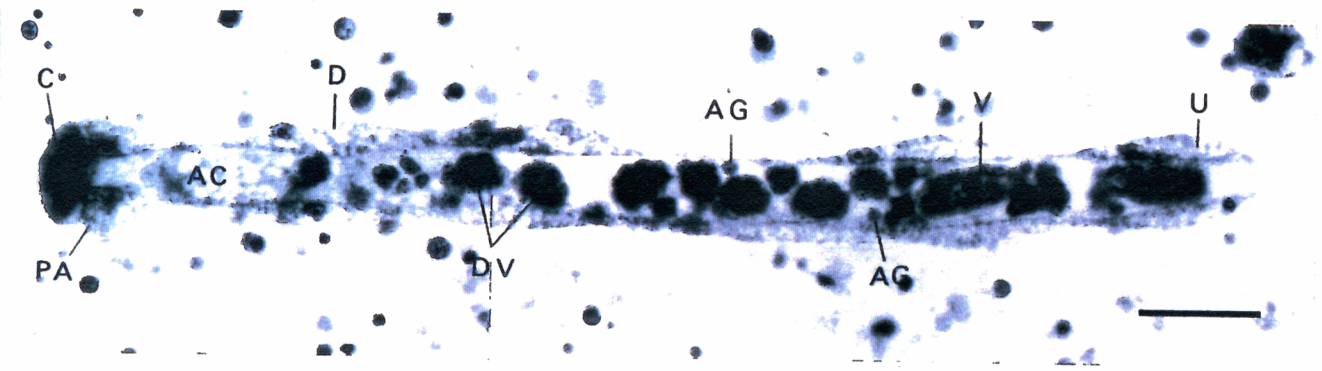


Fig. 1. Light micrograph of a nematogen of Dicyema japonicum. Scale bar represents 50 μm.

Abbreviations for this and subsequent Figures (Figs. 2-4, 6, 8-10): AC, axial cell; AG, agamete; C, calotte; D, diapolar cell; DV, developing vermiforms; M, metapolar cell; N, nucleus of the axial cell of a nematogen; P, propolar cell; PA, parapolar cell; PAC, prospective axial cell; U, uropolar cell; V, fully formed vermiform.

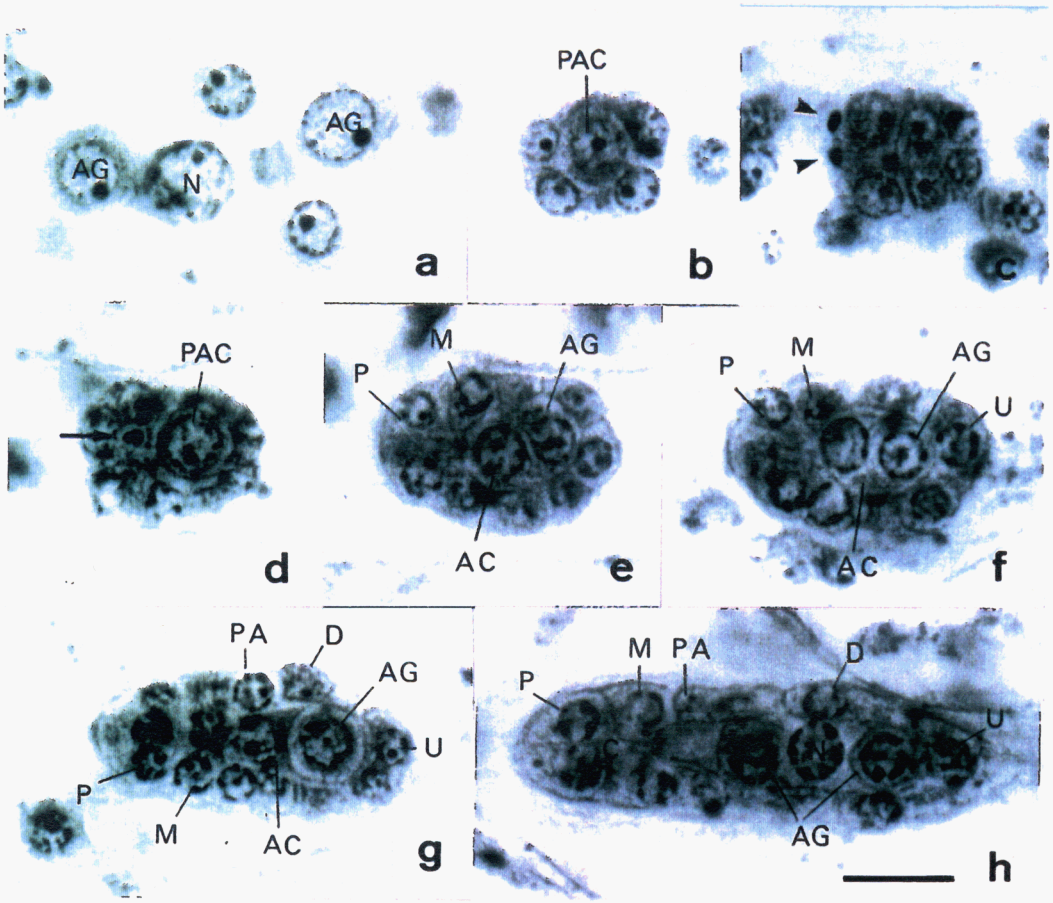
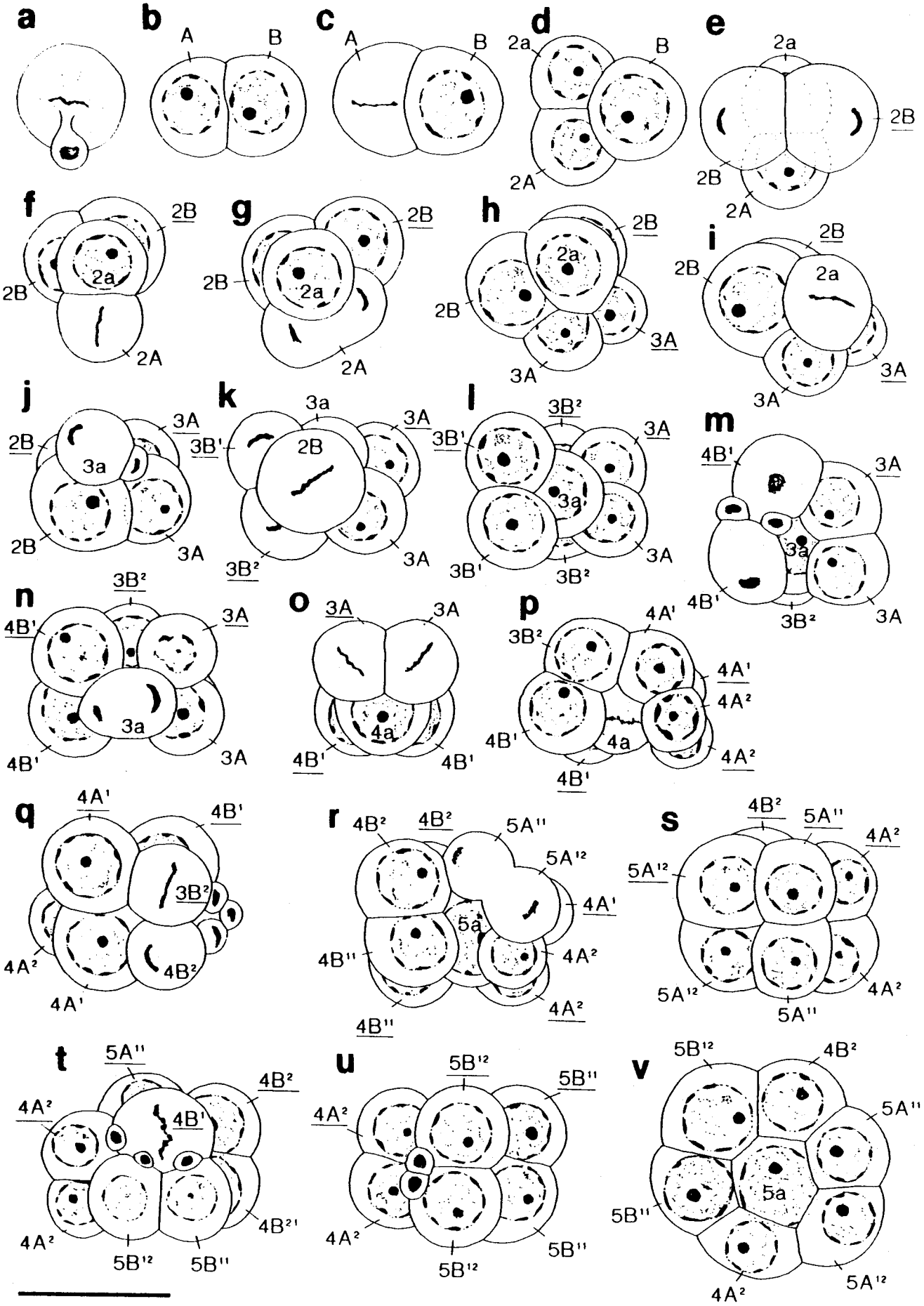


Fig. 2. Light micrographs of developing vermiforms within the axial cells of nematogens of D. acuticephalum. Photographs were taken at a magnification of 2,000 diameters under an oil-immersion objective. Scale bar represents 10 μm. **(a)**: Agametes (AG) and the nucleus of an axial cell of a nematogen (N). **(b)**: Nine-cell stage (optical section). **(c)**: Thirteen-cell stage (surface). The arrowheads indicate degenerating cells produced after extremely unequal divisions of mother cells of peripheral cells. **(d)**: Fifteen-cell stage (optical section). The arrow indicates a degenerating cell produced after an extremely unequal division of the prospective axial cell (PAC). **(e)**-**(h)**: Vermiform embryos (optical section). In (f), an agamete (AG) is incorporated into the cytoplasm of an axial cell (AC).


a
b
A
B
c
A
B
d
2a
B
2A
e
2a
2B
2B
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f
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2B
2A
g
2B
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2B
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h
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i
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j
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3B'
3a
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k
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3A
2B
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l
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4B'
3A
3a
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o
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3A
4a
4B'
4B'
p
4A'
3B²
4A'
4A²
4B'
4a
4A²
4B'
q
4A'
4B'
3B²
4A²
4B²
4A'
r
4B²
4B²
5A''
5A'²
4A'
5a
4B''
4A²
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s
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5A'²
4A²
5A'²
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t
5A''
4B²
4A²
4B'
4A²
4B²'
5B'²
5B''
u
5B'²
5B''
4A²
4A²
5B''
5B'²
v
5B'²
4B²
5A''
5a
5B''
4A²
5A'²

Fig. 3

Fig. 3. Sketches of early embryos of Dicyema acuticephalum. Scale bar represents 10 um. (a): An agamete undergoing an extremely unequal division. This division is not always seen. (b) and (c): Two-cell stage. In (c), a metaphase figure in cell A is depicted. This division produces a prospective axial cell (2a) and a mother cell of peripheral cells (2A). (d) and (e): Three-cell stage. In (e), a telophase figure in cell B is depicted. This division produces a mother cell of the left head (2B) and of the right head (2B). (f) and (g): Four-cell stage. In (f), a metaphase figure in cell 2A is shown. In (g), an anaphase figure in cell 2A is depicted. This division produces a mother cell of the left trunk (3A) and of the right trunk (3A). (h)-(k): Five-cell stage. In (i), a metaphase figure in cell 2a is shown. In (j), a telophase figure in cell 2a is depicted. This extremely unequal division produces cell 3a and a much smaller cell which is destined to die. In (k), the left (2B) and right cell (2B) divide almost synchronously. (l)-(o): Seven-cell stage. In (m), cells $3B^1$ and $3B^1$ undergo an extremely unequal division to produce cells $4B^1$ and $4B^1$ and two much smaller cells which are destined to die. In (n), cell 3a undergoes an extremely unequal division. In (o), metaphase figures in cells 3A and 3A are shown. (p)-(r) Nine-cell stage. In (p), a metaphase figure (lower center) in cell 4a is depicted. In (q), a metaphase figure in cell $3B^2$ (upper right) and a telophase figure (lower right) in cell $3B^2$ are shown. The cell divisions

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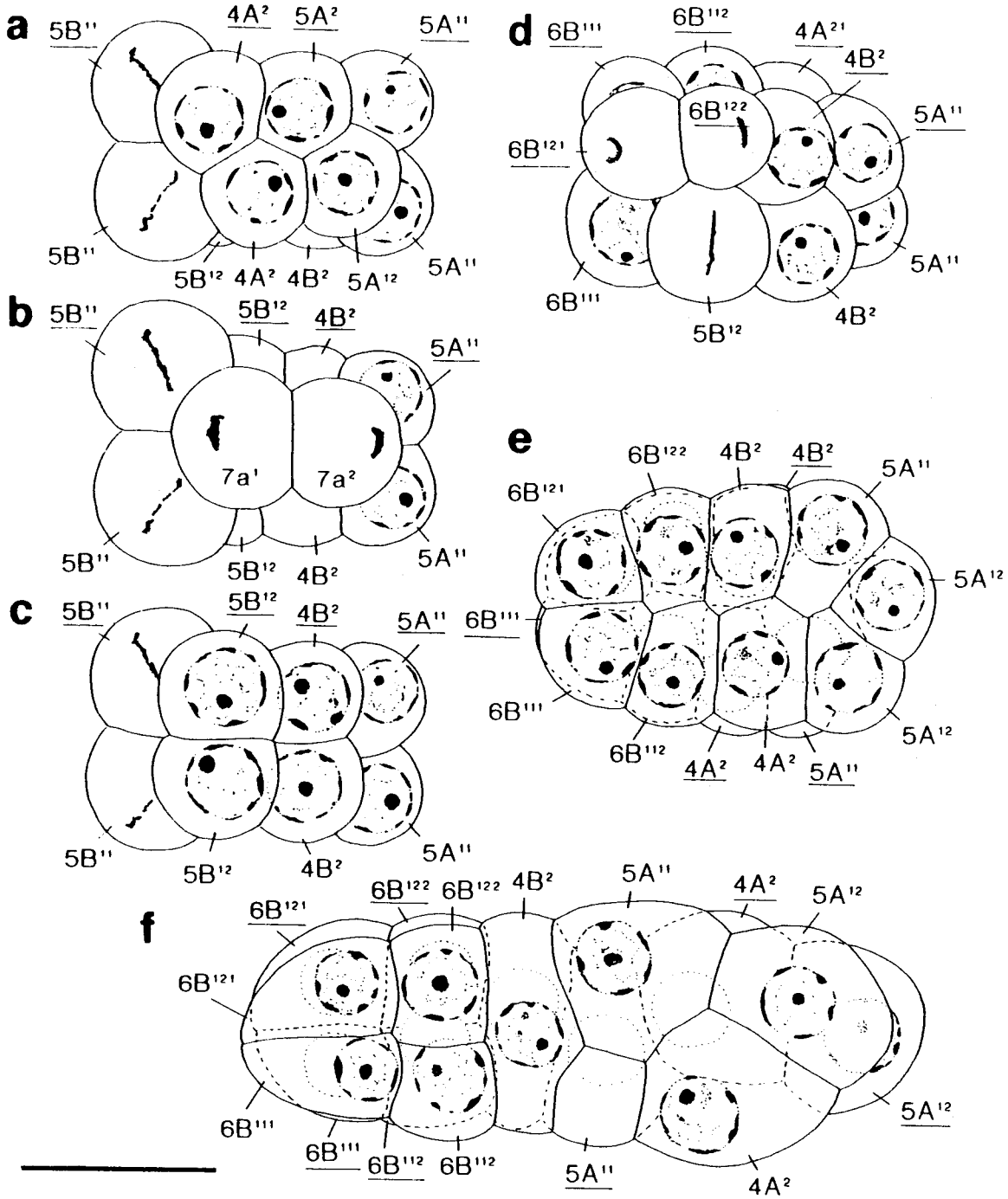
are extremely unequal. In (r), an anaphase figure (upper right) in cell $4A^1$ is shown. This equal division produces cells $5A^{11}$ and $5A^{12}$. (s): Eleven-cell stage. (t): Twelve-cell stage. Note a metaphase figure in cell $\underline{4B^1}$ that is dividing equally to produce cells $\underline{5B^{11}}$ and $\underline{5B^{12}}$. (u): Thirteen-cell stage (surface view). (v): Thirteen-cell stage (sagittal optical section).


a
5B''
4A²
5A²
5A''
5B''
5B'²
4A²
4B²
5A'²
5A''
b
5B''
5B'²
4B²
5A''
7a'
7a²
5B''
5B'²
4B²
5A''
c
5B''
5B'²
4B²
5A''
5B''
5B'²
4B²
5A''
d
6B'''
6B''²
4A²'
4B²
6B'²²
5A''
6B'²'
5A''
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4B²
5B'²
e
6B'²²
4B²
4B²
5A''
6B'²'
5A'²
6B'''
6B'''
6B''²
4A²
4A²
5A''
f
6B'²'
6B'²²
6B'²²
4B²
5A''
4A²
5A'²
6B'²'
6B'''
5A'²
6B'''
6B'''
6B''²
6B''²
5A''
4A²

Fig. 4

Fig. 4. Sketches of late embryos of D. acuticephalum. Scale bar represents 10 um. (**a**): Thirteen-cell stage (ventral view). Note metaphase figures in cells $5B^{11}$ and $\underline{5B^{11}}$ that are dividing equally to produce propolar cells and metapolar cells. (**b**): Thirteen-cell stage (horizontal optical section). Note a telophase figure in cell $6a^{1}$. This division produces an axial cell ($7a^{1}$) and the first agamete ($7a^{2}$). (**c**): Thirteen-cell stage (dorsal view). (**d**): Fifteen-cell stage (dorsal view). Cell $5B^{12}$ divides equally to produce a propolar cell and a metapolar cell. (**e**): Nearly formed embryo (lateral view). (**f**): Fully formed embryo (lateral view). Cilia has been omitted.

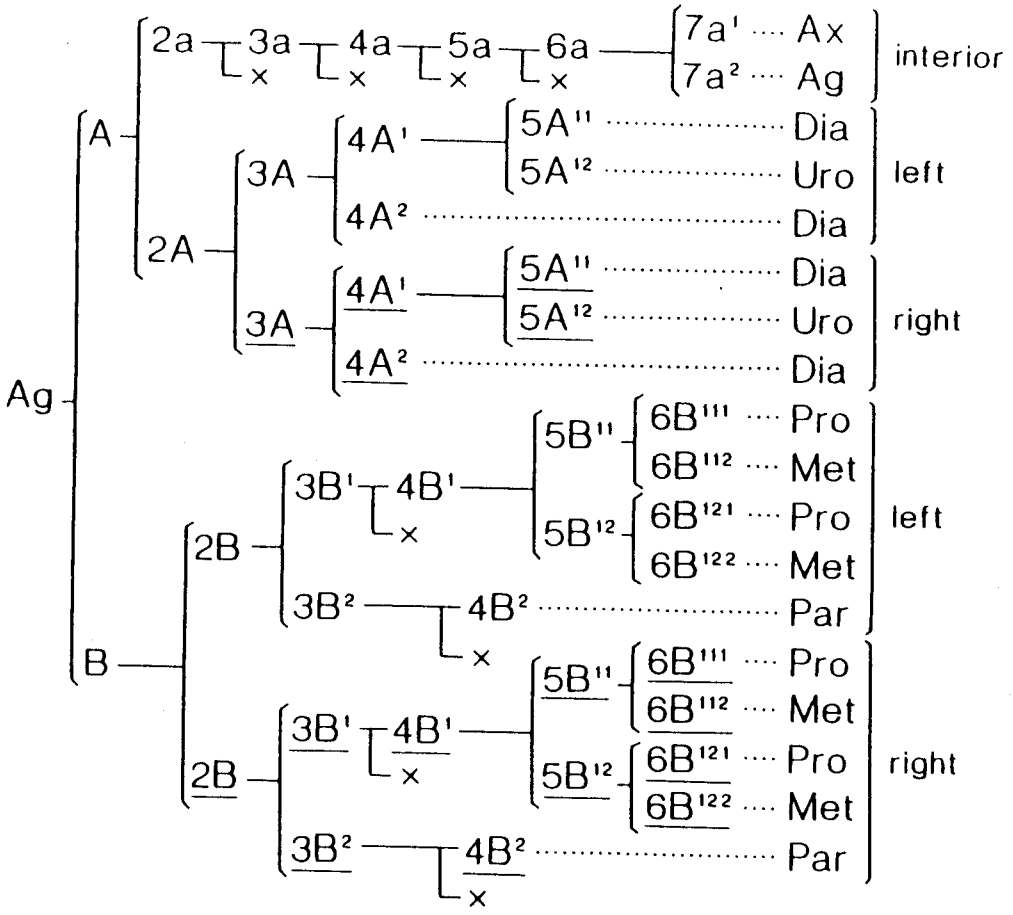

Fig. 5. Cell lineage of the vermiform embryo of Dicyema acuticephalum that has sixteen peripheral cells. A cross (x) indicates that a cell, formed as the result of an extremely unequal division, degenerates and does not contribute to the formation of the embryo.

Abbreviations in Figs. 5, 7, and 11: Ag, agamete; Ax, axial cell; Dia, diapolar cell; Par, parapolar cell; Pro, propolar cell; Met, metapolar cell; Uro, uropolar cell.

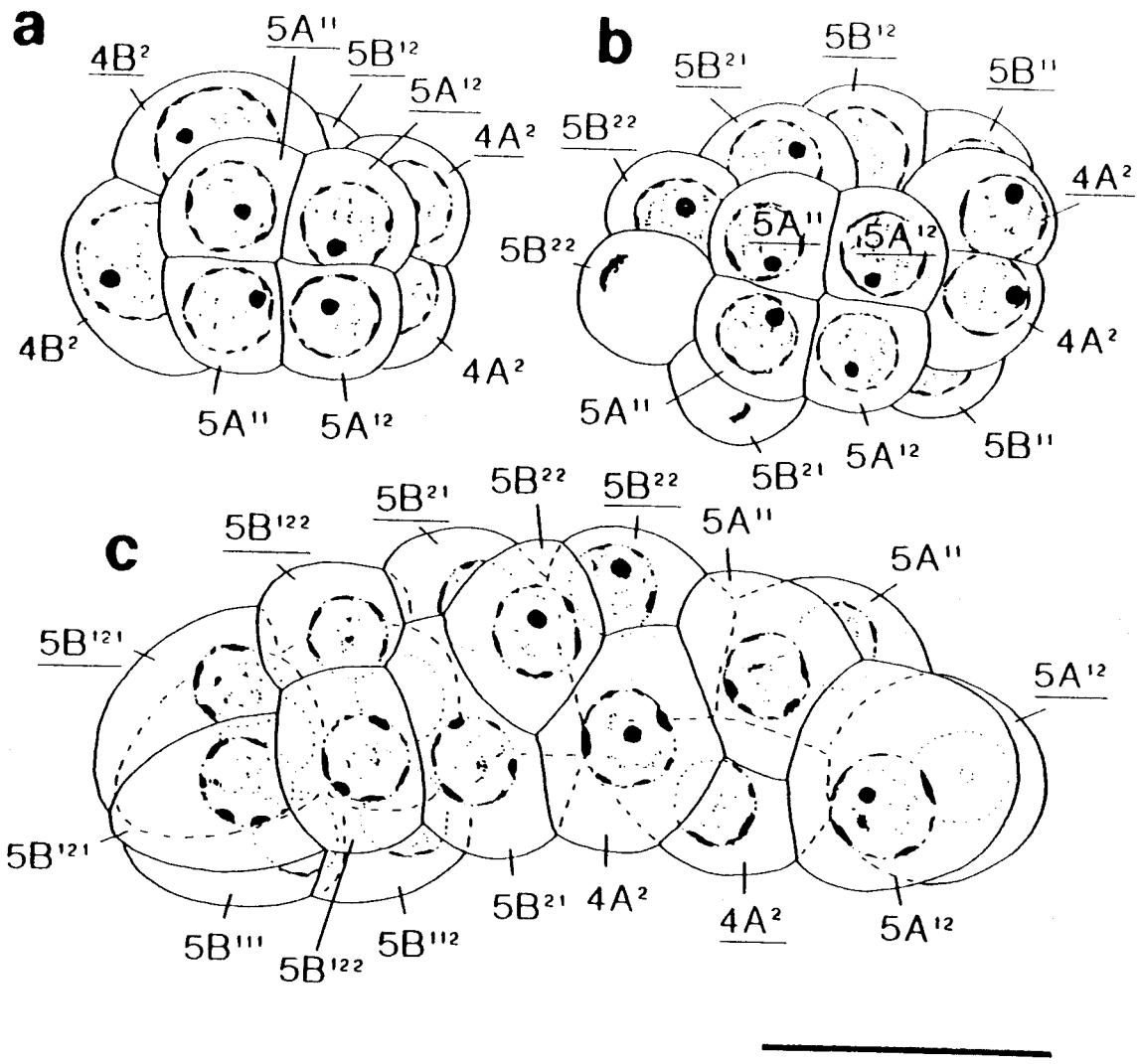

Fig. 6. Sketches of late embryos and a fully formed embryo of Dicyema acuticephalum that has eighteen peripheral cells. Scale bar represents 10 μm. (a): Thirteen-cell stage (from the tail). (b): Fourteen-cell stage (from the tail). Note a telophase figure in cell $4B^2$. This division produces a parapolar cell ($5B^{21}$) and a diapolar cell ($5B^{22}$). (c): Fully formed embryo. Cilia have been omitted.

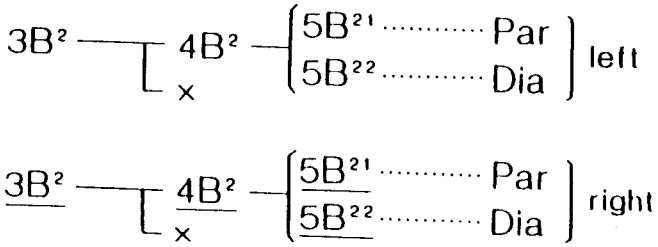

Fig. 7. The lineage of cells $3B^2$ and $\underline{3B^2}$ of the vermiform embryo of Dicyema acuticephalum that has eighteen peripheral cells. The other aspects of cell lineage are the same as those of D. acuticephalum with sixteen peripheral cells. See the legend to Fig. 5 for abbreviations.

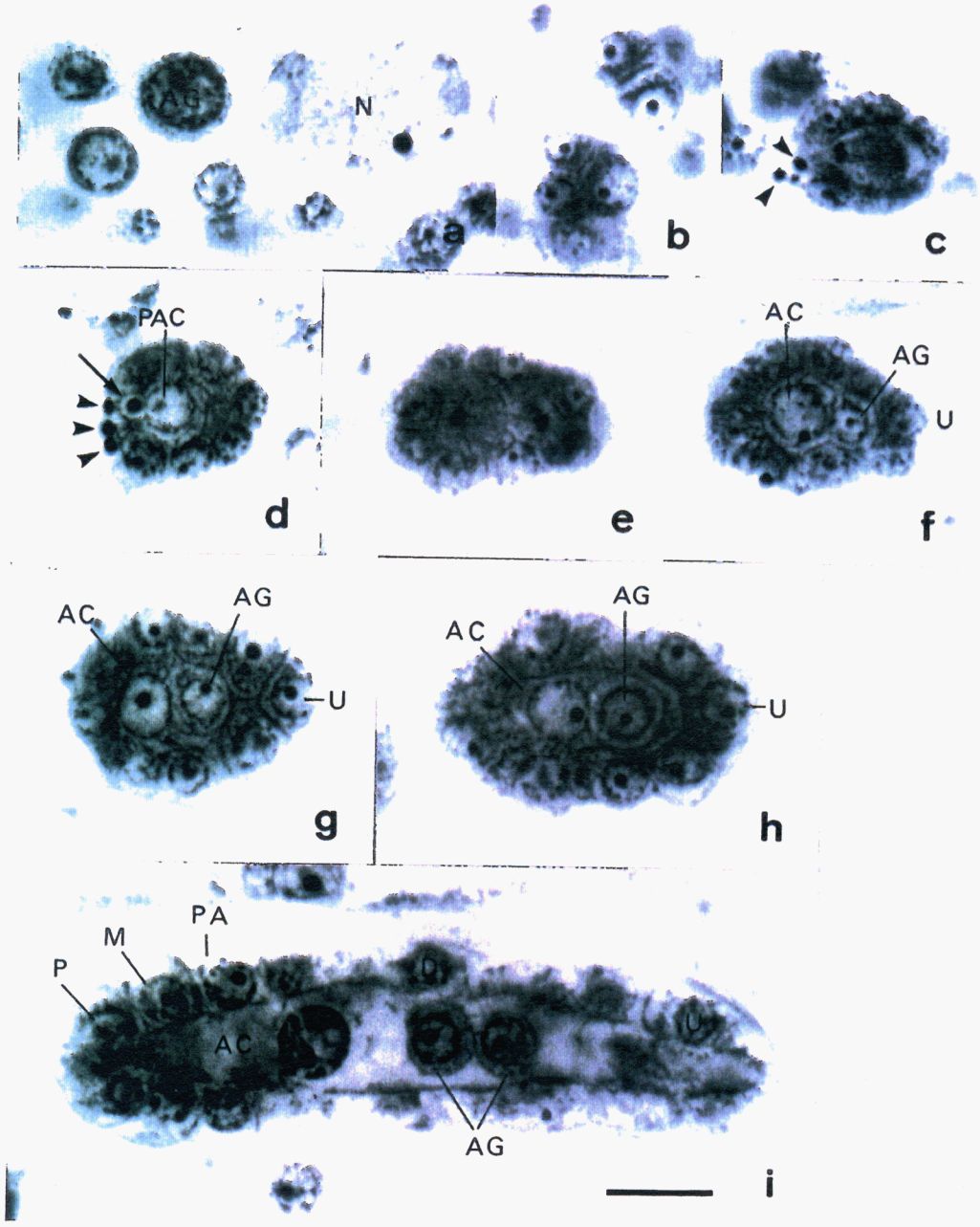

Fig. 8. Light micrographs of developing vermiform embryos within the axial cells of nematogens of D. japonicum. Scale bar represents 10 um. **(a)**: An agamete (AG) and the nucleus (N) of an axial cell of a nematogen. **(b)**: Two-cell stage (upper) and three-cell stage (lower). **(c)**: Thirteen-cell stage (optical section). A prospective axial cell (center) is undergoing an

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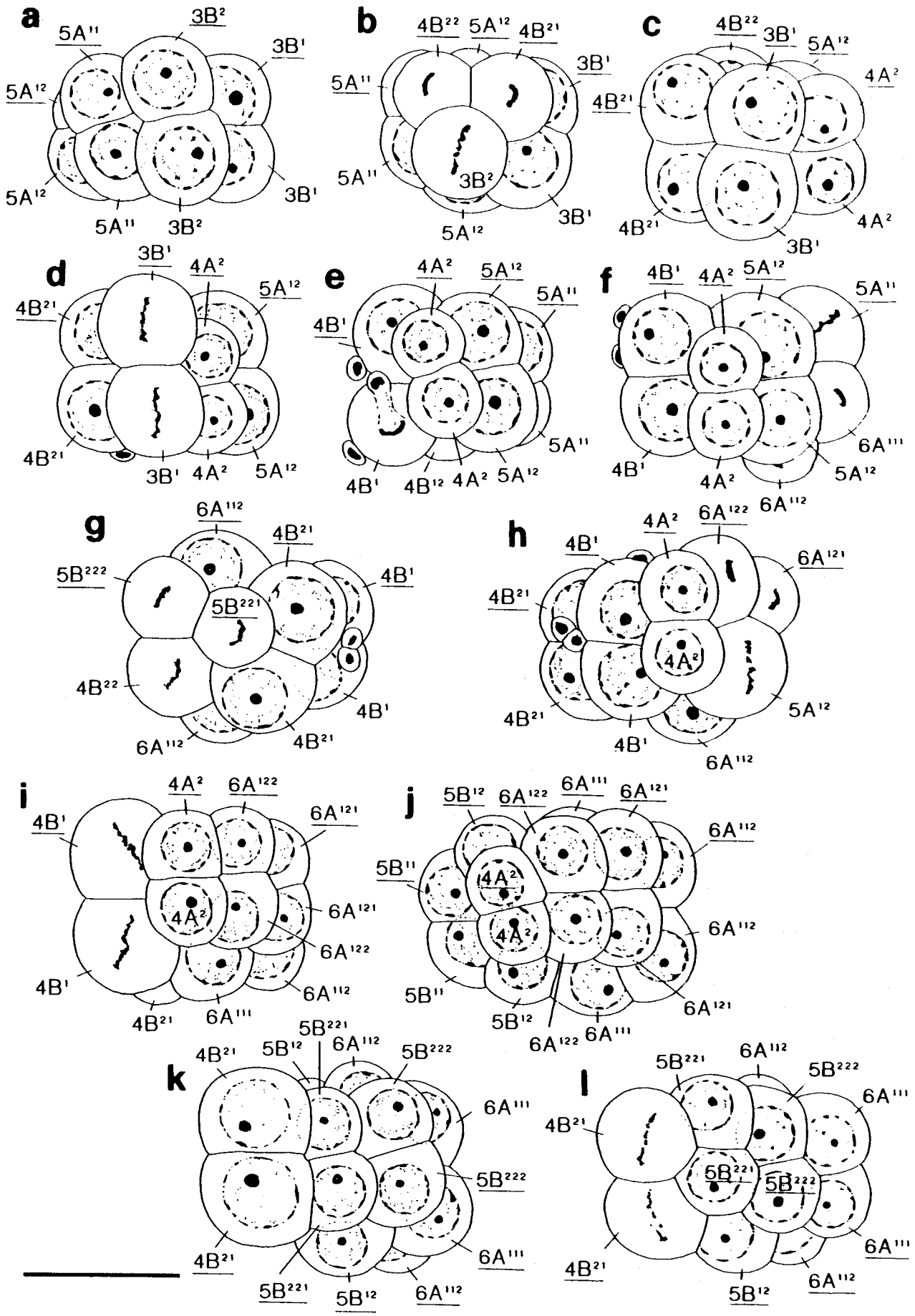
extremely unequal division. The arrowheads indicate degenerating cells produced after extremely unequal divisions. (**d**): Fifteen-cell stage (optical section). The arrowheads indicate degenerating cells produced after extremely unequal divisions of peripheral cells, while the arrow indicates a degenerating cell produced after an extremely unequal division of a prospective axial cell (PAC). (**e**): Seventeen-cell stage (optical section). A prospective axial cell (center) is undergoing an unequal division. (**f**) to (**h**): Developing vermiforms (optical section). In (g) and (h), an agamete (AG) is incorporated in the cytoplasm of an axial cell (AC). (**i**): Fully formed embryo (optical section).


a
5A11
3B2
3B1
5A12
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3B1
5A11
3B2
b
4B22
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5A11
3B2
3B1
5A12
c
4B22
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4B21
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6A112
5B222
6A111
4B21
5B221
5B222
6A111
4B21
6A112
5B12

Fig. 9

Fig. 9. Sketches of embryos of Dicyema japonicum from the eleven-cell to the twenty-one-cell stage. Scale bar represents 10 um. (**a**) and (**b**): Eleven-cell stage. In (b), a telophase figure in cell $\underline{3B^2}$ (upper) and a metaphase figure in cell $3B^2$ (lower) are seen. (**c**)-(**f**): Thirteen-cell stage. In (d), metaphase figures in cells $3B^1$ and $\underline{3B^1}$ are shown. In (e), a later anaphase figure (lower left) in cell $3B^1$ is depicted. This division is extremely unequal and produces cell $4B^1$ and a much smaller cell. In (f), a metaphase figure in cell $\underline{5A^{11}}$ (upper right) and a telophase figure in cell $5A^{11}$ (lower right) are shown. (**g**): Sixteen-cell stage. Note a telophase figure of cell $\underline{4B^{22}}$ (upper left) and a metaphase figure in cell $4B^{22}$ (lower left). (**h**): Seventeen-cell stage. Note a telophase figure of cell $\underline{5A^{12}}$ (upper right) and a metaphase figure in cell $5A^{12}$ (lower right). (**i**): Nineteen-cell stage (ventral view). Note metaphase figures (left) in the $4B^1$ pair. These divisions produce propolar cells ($5B^{11}$ and $\underline{5B^{11}}$) and metapolar cells ($5B^{12}$ and $\underline{5B^{12}}$). (**j**): Twenty-one-cell stage (ventral view). (**k**) and (**l**): Twenty-one-cell stage (dorsal view). In (l), metaphase figures (left) in the $4B^{21}$ pair are shown. These divisions produce propolar cells ($5B^{211}$ and $\underline{5B^{211}}$) and metapolar cells ($5B^{212}$ and $\underline{5B^{212}}$).

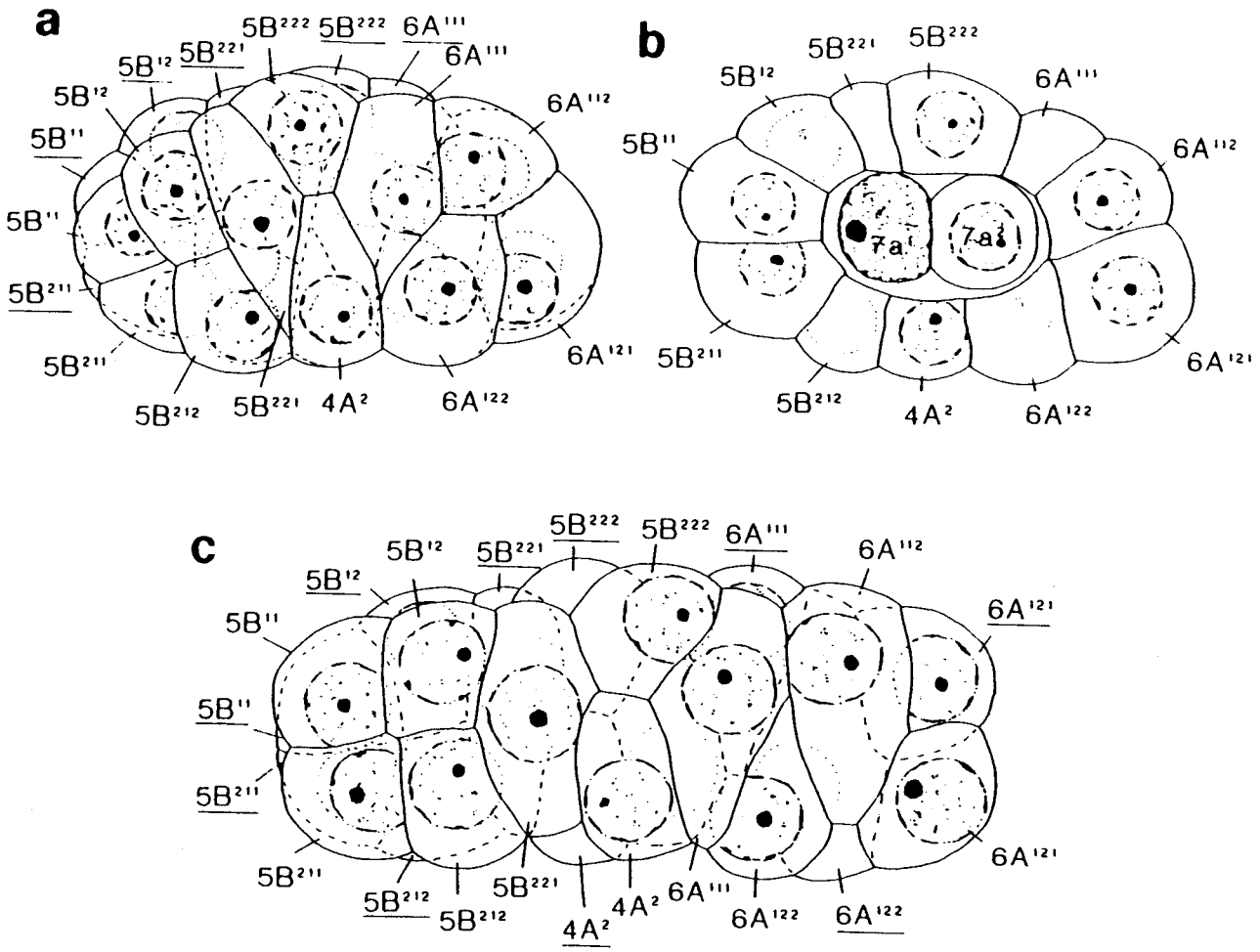

Fig. 10. Sketches of formed embryos of Dicyema japonicum. Scale bar represents 10 μm. **(a)**: Lateral view. **(b)**: Sagittal optical section. Note an agamete ($7a^2$) in the cytoplasm of an axial cell ($7a^1$). **(c)**: A formed embryo (lateral view).

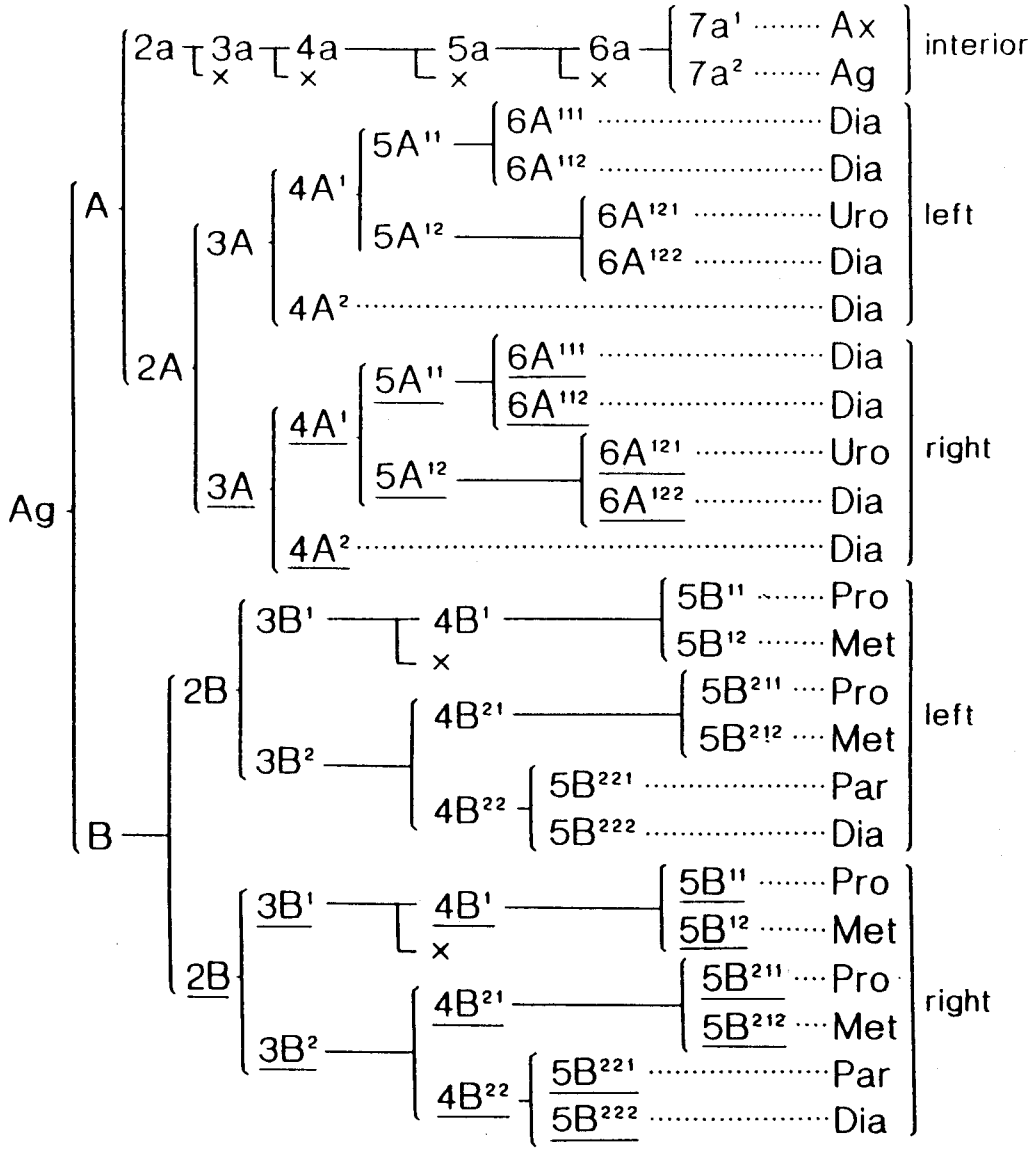

Fig. 11. Cell lineage of the vermiform embryo of *Dicyema japonicum*. See the legend to Fig. 5 for abbreviations.

CHAPTER V

An Attempt to Culture Dicyemid Mesozoans *in vitro*

INTRODUCTION

Dicyemid mesozoans are found in the renal sac of benthic cephalopod molluscs. The bodies of dicyemids consist of only 20 to 40 cells and they are organized very simply [9, 10, 11]. Two kinds of adult forms, nematogens and rhombogens, are found in dicyemids. Asexual reproduction occurs within the axial cell of nematogens and a vermiform embryo develops from an agamete (axoblast). The process of embryogenesis of vermiforms has been recently described in detail [4]. On the other hand, sexual reproduction takes place within the axial cell of rhombogens. A hermaphroditic gonad, which is called an infusorigen, is formed within the axial cell of rhombogens and fertilization occurs around the infusorigen. The zygote undergoes cleavages and develops into an infusoriform embryo within the axial cell. The processes of gametegenesis and cleavage have also been described in detail recently [1, 3].

The life cycle of dicyemids has been well studied, but one important aspect is still unknown. Thus, the developmental process from a germinal cell of the infusoriform embryo to the stem nematogen has not been elucidated. If *in vitro* culture system of dicyemids is established, we could observe development of the germinal cells and obtain full knowledge of the life cycle of these intriguing animals. Although some attempts to culture dicyemids have been made, no substatial information about their life cycle has been obtained [6, 7, 13].

The embryos of dicyemids are consistently composed of a very small number of cells. These cells, with more or less clear

evidence of specific differentiation, are produced after only a few rounds of cell division. The development of the embryos of dicyemids appears to be the simplest type of the development seen in the animal kingdom. Thus, these embryos might be useful as the simplest model system for the study of cell differentiation and morphogenesis in animals, if in vitro culture method becomes available.

In this study, we examined how long nematogens and infusoriform larvae can survive in the octopus urine and in seawater. We also attempted to culture them in an artificial medium.

METERIALS AND METHODS

Animals

Octopus vulgaris, purchased from fish markets, was the primary source of dicyemids. Three species of dicyemids usually found in Octopus vulgaris, namely, Dicyema acuticephalum, Dicyema japonicum, and Dicyema misakiense, were used in this study. D. acuticephalum is a small sized dicyemid, rarely exceeding 1 mm in length [12]. D. japonicum and D. misakiense are medium sized dicyemids, rarely exceeding 1.5 mm [2].

Isolation of Dicyemids from the Host

The body of host cephalopods was separated from the tentacls just beyond the eyes. The body was tightly bound up with a

string at the level of the eyes. The mantle was inverted and then the body was briefly washed in 70% ethanol three times. The external surface of the renal sac was washed off again with 70% ethanol and it was promptly wiped off with a sterile filter paper. A small incision was made in the wall of the renal sac and the urine containing dicyemids was sucked up with a sterile Pasteur pipette from the incision. Immediately after removal, the urine was transferred into a beaker with a sterile sea water containg 500 units of penicillin and 0.5 mg of streptomycin per milliliter.

The suspension was centrifuged for one minute at 100-200 x g. After centrifugation, the supernatant was withdrawn and replaced in a beaker with an equal volume of the antibiotics-containing seawater and the dicyemids were resuspended by gently shaking. The centrifugation and resuspention were repeated three times. After the final resuspention, the supernatant was withdrawn and replaced in a beaker with an equal volume of one of culture media; urine, seawater, or artificial medium.

Preparation of the Seawater and the Urine

The natural seawater was filtered through a filter paper and it was then sterilized by filtration through a 0.2 μm millipore filter. The pH was adjusted to 6.8 by 1N HCl. The urine was obtained from host octopuses and was diluted by adding the same volume of sterile seawater containing 100 units of penicillin and 0.1 mg of streptomycin per milliliter. The diluted urine was used after filtration through a 0.2 μm millipore filter.

Preparation of the Artificial Culture Medium

The culture method first described by Lapan and Morowitz [7] was used with some modification. Stock solutions were made according to Tables 1 and 2, and were stored frozen. Other components are weighed at the time of preparation of the medium (Table 3). RPMI medium 1640 was obtained commercially from Gibco BRL Life Technologies, Inc, New York, and was free of sodium phosphate. Amino acids were supplied by basal medium Eagle without glutamine and vitamins were supplied by basal medium Eagle. These were obtained commercially from Flow Laboratories, Mclean Virginia. Other chemicals were from Wako Pure Chemical Industries, Ltd. Each components, except for fetal bovine serum, were added to 500 ml flask and dissolved in about 500 ml of sterile seawater. The medium was filtered through a 0.2 μm millipore. Finally, fetal bovine serum, which obtained commercially from Gibco BRL life Technologies, was added, because in a prvious report better survivial was obtained when the serum was not filtered [7]. The medium was diluted to one litter with sterile seawater.

Incubation

Nunclon 4-well multidishes from Nunc were used, because they are suitable for microscopical observations. Isolated dicyemids were placed in culture media in 4-well multidishes. The medium was inoculated to have a minimum concentration of 10^3 vermiforms

per milliliter [7]. Culture dishes were kept at 14-15°C in the dark in the low and constant temparature chamber. Microscope observations were conducted under a minimal level of light. When a ciliary activity decreased noticeably, the trial solution, namely, the urine, the seawater, or the medium, was changed. Thus, the exchange was done in every three to four days.

RESULTS

The survival times of nematogens and infusoriform larvae in vitro in the octopus urine and in seawater are shown in Fig. 1. The nematogens have been alive more than for ten days in the urine, but they died within five days in seawater. The infusoriform larvae became immobile only within two days in both the urine and seawater. In some circumstances, ciliary activity ceased but the organisms did not lyse or shrink for some days. It was, thus, difficult to determine whether the organisms are dead or not in these cases, but high ciliary activity is certainly an indication of healthy condition and this activity was taken as a criterion whether they are alive or not.

In the artificial medium, the infusoriforms became immobile only within two to three days as in the case the urine and sea water. However, the individuals of vermiform stages, namely, nematogens, rhombogens, and vermiforms, have survived well up to three weeks (Fig. 2). Beyond three weeks, unusual individuals appeared; some became twisted (Fig. 3), others were knotted (Fig. 4). After a month in culture, the ciliary activity became

gradually low and the outer surface of peripheral cells, except for a calotte, transformed unevenly. Some dicyemids lost the peripheral cells and their axial cell were naked (Fig. 5). During this in vitro culture, two kinds of dicyemid embryos, namely, vermiform embryos and infusoriform embryos could develop within axial cells of nematogens and rhombogens, and were escaped from the axial cells into the medium (Figs. 6 and 7).

After infusoriforms were immobile, their four urn cells were expelled and were frequently seen on the bottom of the culture well (Fig. 8). Even after the infusoriform body was lysed, the urn cells have remained intact about for two days (Fig. 9).

Many stellate cells often proliferated on the bottom of the culture wells (Fig. 10). These cells are probably derived from the host lymph. The dicyemids survived very well and lived for six weeks, these cells proliferated.

DISCUSSION

In this study, it was only for two days that infusoriforms can swim in the urine and sea water in vitro. The infusoriforms, therefore, might have rather short periods to swim in the sea, although the time in vitro may not be necessarily reflected in the natural environment. The fate of infusoriforms that escaped the host body has not been followed to date, although there is some experimental evidence that no secondary host is required and the infusoriform directly infects a new host [5]. Infusoriforms should find a new host quickly in the sea, if they can swim only

for two days even in the sea. It seems probable that they could not move far from the area where they escaped the cephalopod body. Nevertheless, the percentage of infection is almost one hundred per cent in nature. Since the population density of infusoriforms in the ocean is assumed to be low, it is plausible that the infusoriforms may infect new cephalopods favorably at the time when cephalopods are close together in their breeding season.

The dicyemids did not survive longer in the present artificial medium then in the original method tried by Lapan and Morowitz [7]. According to Lapan [8], dominant low molecular weight substances of the renal tissue of octopuses are homarine, tryptophan, and hypoxanthine. These all appear to be essential for dicyemids. Arginine or some of other amino acids of urea cycle are also required in substantial concentrations [8]. In this study, homarine (N-methyl picolinic acid) could not be obtained and some inferior survival time in this study may be due to the absence of homarine.

The stellate cells, which are probably derived from the host lymph, often proliferated on the bottom of the culture well. The survival of dicyemids was very well, when thses cells were proliferating. If a large number of these cells could be cultured, the dicyemids might be kept much better.

McConnaughey [9] observed expulsion of four urn cells from the infusoriform larva. This phenomena were also frequently seen in the present study. The urn cells have remained intact about for two days, even after the body of the infusoriform was broken down. Lapan and Morowitz [7]observed asymmetric division of the

urn cell on the bottom of the culture flask following lysis of the larva. This event may be a step for further development. The development appears to culminate in the first vermiform of a new population and that is a stem nematogen [5, 9, 10, 11]. However, this developmental process and the significance of the stem nematogen are still under some controversy. This developmental process should further be studied *in vitro* to obtain a complete knowledge of the life cycle of dicyemids.

SUMMARY

Survival times of nematogens and infusoriform larvae *in vitro* in the octopus urine and in seawater were studied. Nematogens have been alive more than for ten days in the urine, but they died within five days in seawater. The infusoriform larvae became immobile only within two days in both the urine and seawater. Dicyemids were also cultured *in vitro* in an artificial medium. They could be kept for over one month. During *in vitro* culture, two kinds of embryos, vermiform embryos and infusoriform embryos, could develop within the axial cells of nematogens and rhombogens, and they escaped into the medium apparently in a healthy condition.

ACKNOWLEDGMENTS

I would like to express my sincere thanks to Dr. Kazuo Ito and Dr. Yasuo Nakanishi for their valuable advice and encouragement during the course of this study.

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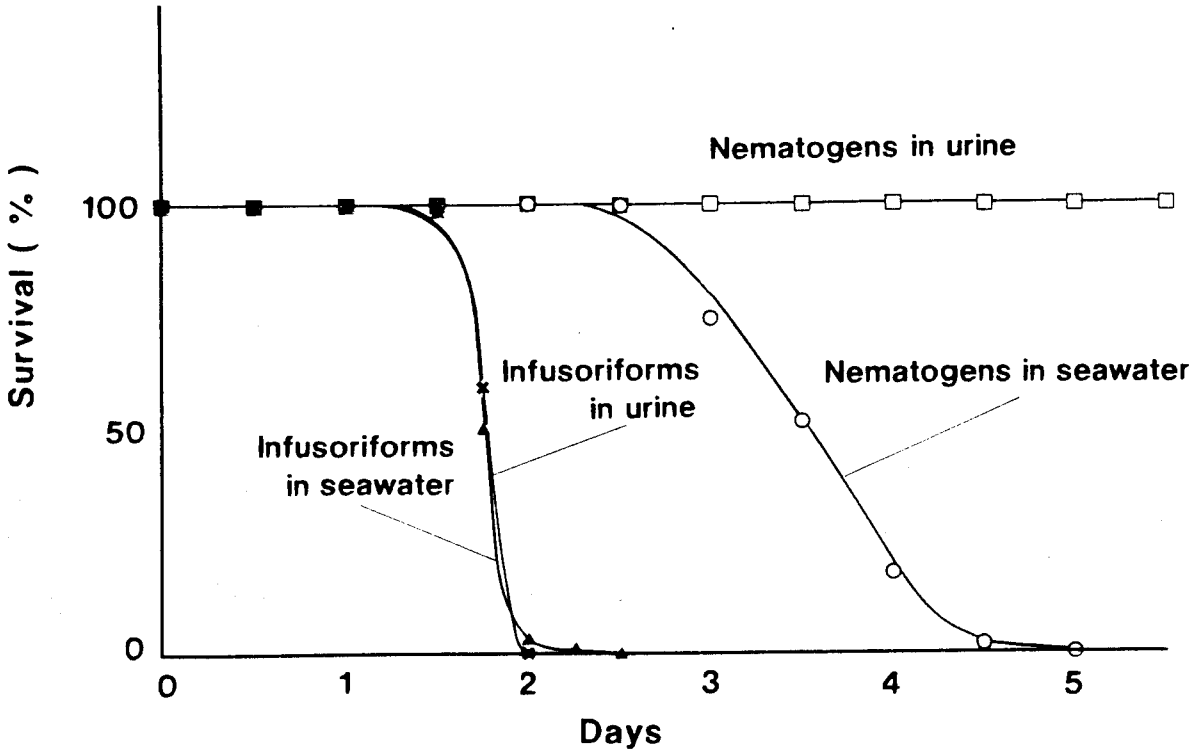

Fig. 1. The survival of nematogens and infusoriform larvae of Dicyema japonicum in vitro in the octopus urine and in seawater at 14°C.

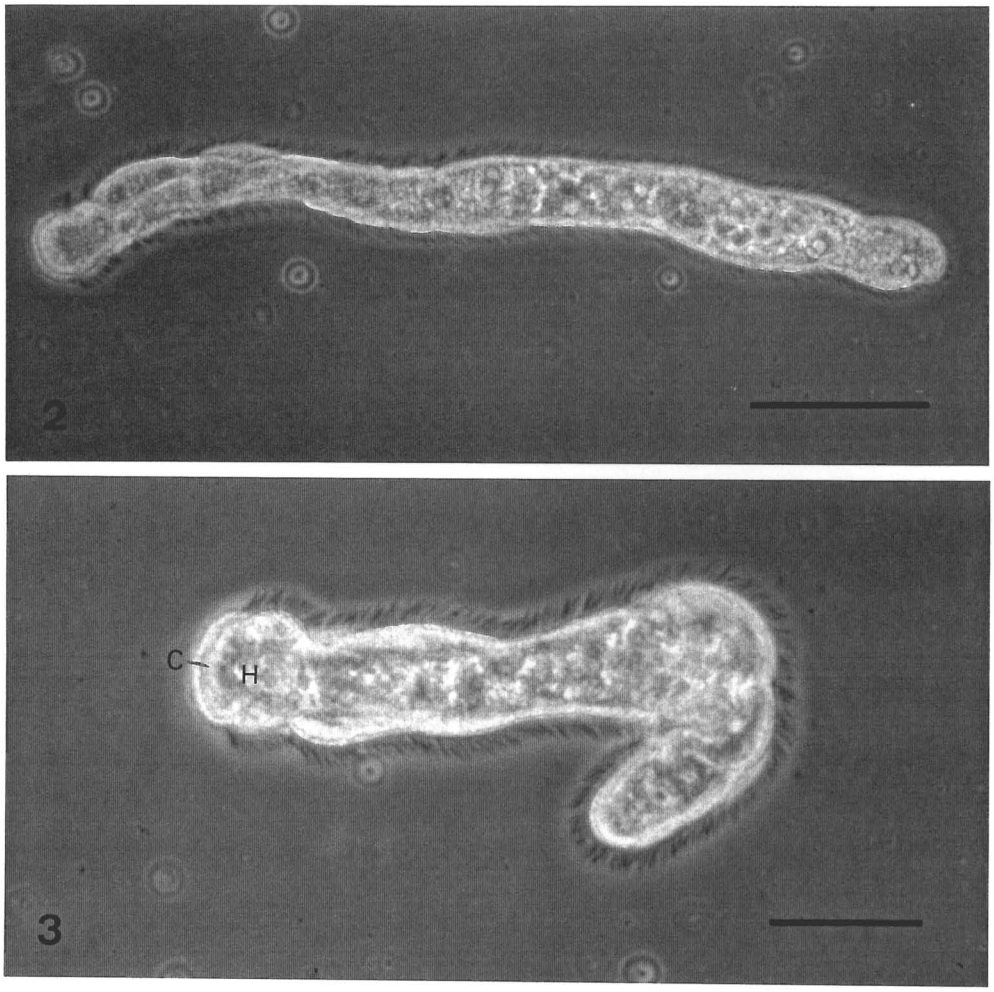

Fig. 2. A rhombogen of Dicyema japonicum cultured for three weeks. The picture was taken under a phase contrast microscope. Scale bar represents 100 um.

Fig. 3. A rhombogen of Dicyema japonicum cultured for four weeks. The picture was taken under a phase contrast microscope. Scale bar represents 50 um. C, calotte; H, head.

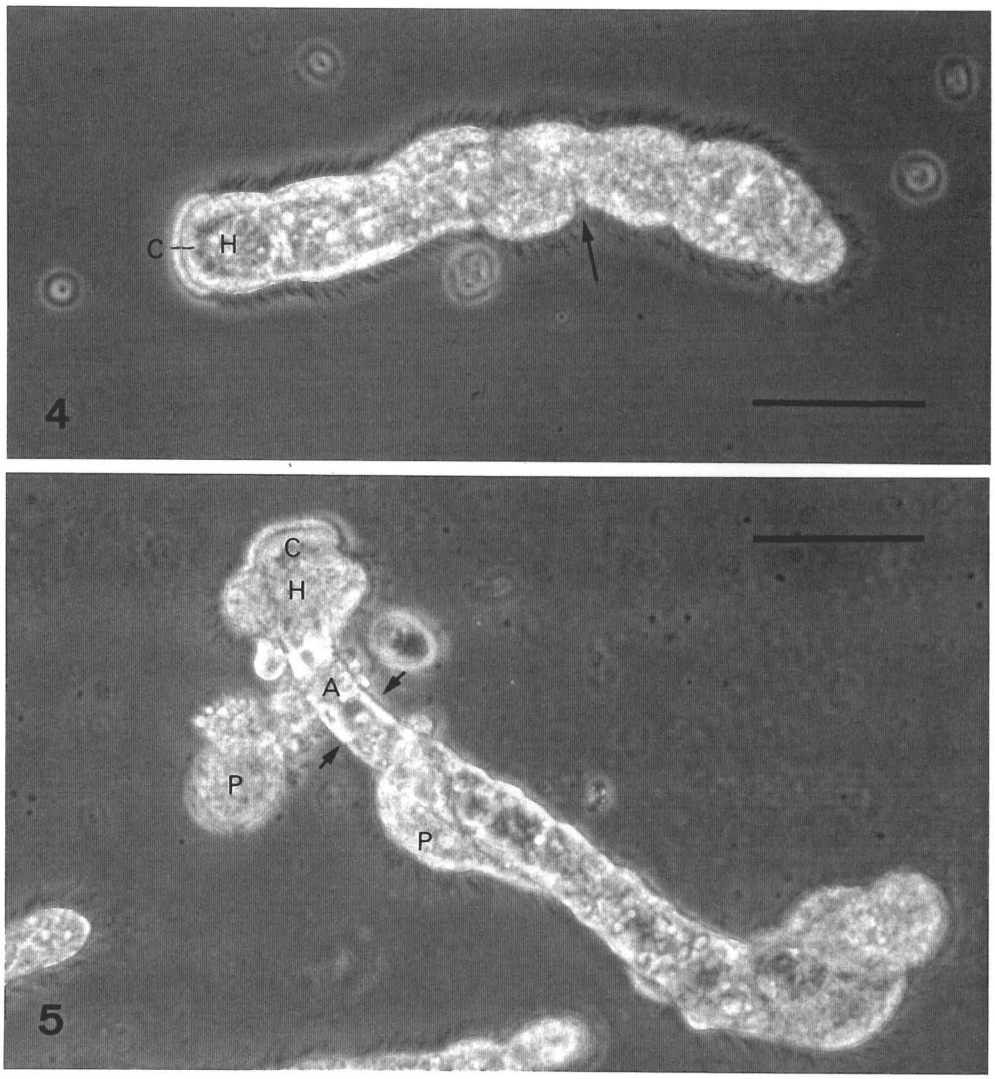

Fig. 4. A rhombogen of Dicyema japonicum cultured for four weeks. The picture was a taken under phase contrast microscope. Scale bar represents 50 um. The arrow indicates a knotted point. C, calotte; H, head.

Fig. 5. A rhombogen of Dicyema japonicum cultured for a month. The picture was a taken under a phase contrast microscope. Arrows point to regions where the axial cell is exposed. Scale bar represents 50 um. A, axial cell; C, calotte; H, head; P, peripheral cell.

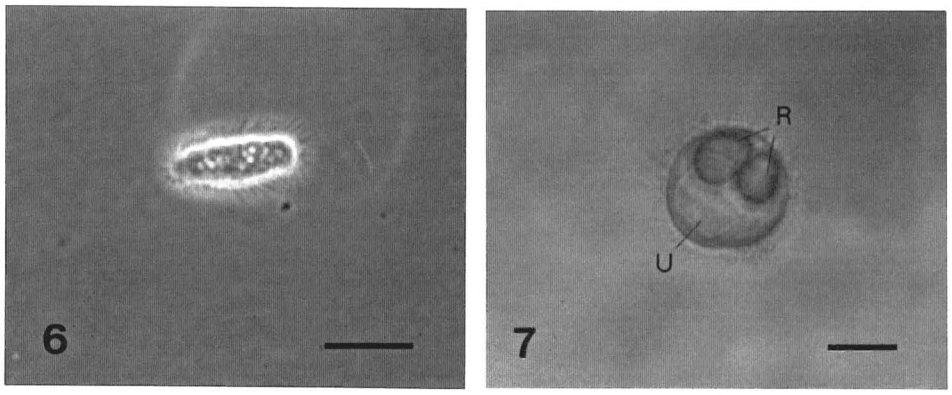

Fig. 6. A vermiform larva of Dicyema japonicum in culture medium. The picture was taken under a phase contrast microscope. Scale bar represents 50 um.

Fig. 7. An infusoriform larva of Dicyema japonicum in culture medium. The picture was taken under a phase contrast microscope. Scale bar represents 10 um. R, refringent body; U, urn.

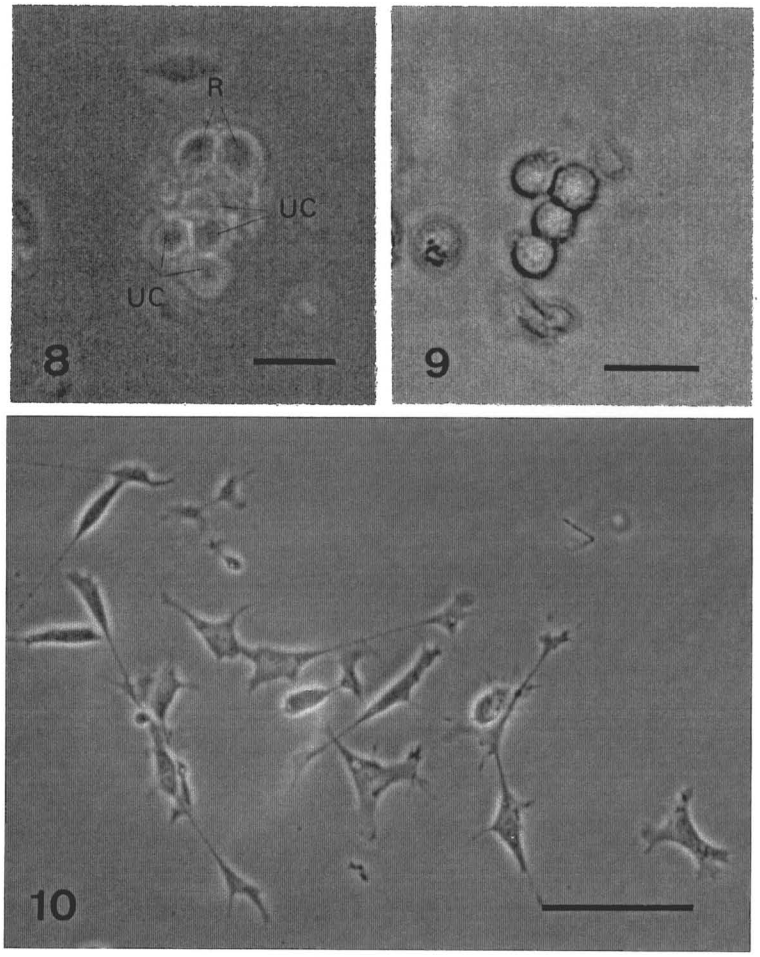

Fig. 8. Expelled four urn cells from the infusoriform embryo of Dicyema japonicum on the bottom of the culture well. The picture was taken under a phase contrast microscope. Scale bar represents 10 um. R, refringent body; UC, urn cell.

Fig. 9. Four urn cells remaining after the infusoriform body was lysed to be disappeared. Scale bar represents 10 um.

Fig. 10. Stellate cells proliferating on the bottom of the culture well. Scale bar represents 50 um.

Table 1 Stock solution 1 of purines and pyrimidines

Compound	mg/100 ml
Adenine	50
Guanine	50
Hypoxanthine	500
Xanthine	25
Cytosine	50
Thymine	25
Uracil	30
HCl (11N)	15 ml

Table 2 Stock solution 2 of other additives

Compound	mg/100 ml
Guanosine 5' monophosphate	20
Uridine 5' monophosphate	20
D-Glucosamine HCl	100
N-Acetyl Glucosamine	20
Glucose-1-Phosphate	50

Table 3 Preparation of medium

Additive	mg/l	Additive	ml/l
Glucose	500	Sterile seawater	500
Fucose	10	Solutions 1 and 2 each	10
Mannose	100	RPMI 1640 medium	20
Ribose	50	Amino acids	10
		Vitamins	10
Taurin	75	(Filter and sterilize)	
Sodium pyruvate	20	(Complete to one liter	
Ascorbic acid	2	with sterile seawater)	
Streptomycin	100	Fetal calf serum	20
Penicillin	10^5 I.U./l	(Adjust pH to 6.8 by NaOH)	

DISCUSSION

The general biology of dicyemids was studied by two distinguished pioneers, Nouvel [28, 29] and McConnaughey [25, 26, 27]. However, there are still many problems to be answered. The present study has given a basis for the systematics of Japanese dicyemids and brought about detailed knowledge of their morphology and embryology.

Dicyemid Fauna in Seven Cephalopod Species

I have examined the dicyemids in the renal sacs of seven species of Japanese cephalopods, namely, Octopus vulgaris, Octopus minor, Octopus fangsiao, Octopus dofleini, Sepioteuthis lessoniana, Sepia esculenta, and Sepia lycidas. Nineteen dicyemid species, including four described species, were observed in their renal sacs as follows: in Octopus vulgaris, four species of the genus Dicyema (including D. acuticephalum and D. misakiense); in Octopus fangsiao, three species of the genus Dicyema; in Octopus minor, three species of the genus Dicyema; in Octopus dofleini, one species of the genus Dicyemennea; in Sepia esculenta, two species of the genus Dicyema, one species of the genus Pseudicyema (P. truncatum), and three species of the genus Dicyemennea; in Sepia lycidas, one species of the genus Dicyema; in Sepioteuthis lessoniana, one species of the genus Dicyema (D. orientale). In this study, two species, Dicyema japonicum sp. nov. from Octopus vulgaris and Dicyema clavatum sp. nov. from

Octopus minor, were described in detail. The other thirteen species are not fully characterized yet. Species of the genus Dicyema were found in all cephalopod species examined, except for Octopus dofleini, and species of the genus Dicyemennea were found in Octopus dofleini and Sepia esculenta. In the world, more than half of the described species belong to the genus Dicyema and about one third of the described species belong to the genus Dicyemennea. In the Japanese cephalopod species examined, the genus Dicyema is also a dominant genus.

Two to five dicyemid species were detected in Octopus vulgaris, Octopus fangsiao, Octopus minor, and Sepia esculenta, while a single species was seen in Octopus dofleini, Sepioteuthis lessoniana, and Sepia lycidas. In early records [25, 28], a single dicyemid species was described from some different cephalopod species, but recently such cases have not been reported. Thus, a single dicyemid species may not infect more than one cephalopod species. There seems to be a specificity between dicyemid parasite and cephalopod host. If the host specificity is definitely confirmed, this relationship may give valuable information to the cephalopod taxonomy.

Dicyemids are believed to infect only benthic or epibenthic cephalopods [12, 26, 28]. In my preliminary study, Watasenia scintillans and Sepiella japonica were not infected by dicyemids. Watasenia scintillans is a pelargic squid. The ecological habitat of Sepiella japonica is unknown. There are still many interesting Japanese cephalopods, e. g. Opisthoteuthis depressa, which are benthic and are not yet studied in terms of dicyemid parasites. The dispersal larvae (infusoriforms) of dicyemids

possess dense bodies in the apical cells and appear to swim down to the bottom of the sea. These features may explain why only benthic and epibenthic cephalopods are infected.

General Consideration of Morphology and Embryology

Dicyemids have much simpler body organization than other multicellular animals, as expressed in the small number of cells which differentiate only into four foundamental types in vermiform stages. According to fine structural studies on dicyemids [24], internal surfaces of peripheral cells, which are adjacent to the plasma membrane of the axial cell, are free of basal lamina. No typical desmosomes or other junctional complexes are seen between two adjacent lateral membranes of the peripheral cells and between the peripheral cell membrane and the axial cell membrane. Although dome-like cavities are observed sporadically between the axial cell and the peripheral cell, little extracellular material is seen in thee cavities. Only a limitted amount of dense material accumulates in a restricted region on the cytoplasmic side of the lateral membrane of the peripheral cells. Thus, the space between the two membranes is only 20 nm wide and appears homogeneous. This may be the simplest fashion of the cell adhesion system seen in multicellular animals. These fine structural observations suggest that the dicyemid is indeed a very simple and minimally differentiated a primitive organism among multicellular animals.

In dicyemids, there is no germ layer and groups of cells are

roughly distinguished only as outer cells and inner cells. In the infusoriform embryos, the outer cells, which occupy mainly the surfaces of the embryo, are derived from the blastmeres of the animal hemisphere of the embryo, and the inner cells are derived from the cells that form the vegetal pole. In the development of infusoriforms, the cell division proceeds spirally in the early stage and later becomes bilateral. The formed embryos finally exhibit bilateral symmetry. These processes of development and cellular rearrangement are observed in many other lower groups of animals and appear to represent the basic pattern of the early development of animals.

However, the developmental process of dicyemids is so simple that it may represent one of the simplest types of development seen in the animal kingdom. In embryogenesis of infusoriforms and vermiforms, cleavages or cell divisions appear to be determinate and result in an embryo with a definite number and arrangement of cells. Unequal divisions occur in certain points of certain cell lines and produce variously sized cells; the largest cells are axial cells of vermiforms and infusorigens, and the smallest cells are germinal cells and spermatogonia. Unequal divisions may contribute to the morphogenesis and cell differentiation. Peculiar and probably programed cell death also can be seen in the development of infusoriforms and vermiforms. Moreover, McConnaughey [26, 27] and Nouvel [28] found that embryos of stem nematogens include a number of degenerating cells. Thus, cell death during the embryogenesis appears to be a constant and general feature of the development of dicyemids. In the embryogenesis of infusoriform embryos, a cell line that

includes cell death produces remarkably differentiated cells, such as capsule cells. Although I can offer no reasonable explanation at present, cell death might somehow be involved in an accelaration of cell differentiation. As a whole, the development of dicyemids seems to be highly programmed. In such a development, there is apparently little plasticity in such a development probably due to the simple body organization of these organisms. During embryogenesis, the dicyemids show some distinctive features such as presence of cells that are incorporated into the other cell cytoplasm. These peculiarities also may be interpreted as specialized conditions or adaptations due to the simple body organization.

Although several contributions were made by Hartmann [9], Lameere [20-22], Nouvel [28, 29], McConnaughey [25-27], and Lapan and Morwitz [23], knowledge of the complete life cycle of dicyemids remain to be clarified. It is unknown how infusoriforms infect the new host and develop into so-called stem nematogens. McConnaughey [26] observed expulsion of four urn cells from the infusoriform larva. In the present study, the urn cells remained intact about for two days, even after the infusoriform body was lysed. Moreover, Lapan and Morowitz [23] observed asymmetric division of the urn cell on the bottom of the culture flask following lysis of the larva. This event may be a step for further development. This developmental process should further be studied <u>in vitro</u> to obtain a complete knowledge of the life cycle of dicyemids.

Phylogenetic Consideration

Although several zoologists have attempted to clarify the phylogenetic position of the dicyemids, it appears difficult to trace their derivation from, or to relate them to, any other phyla. Some zoologist regarded their simple body organization as a result of parasitism [3, 8, 9, 22, 29, 35], while others considered that dicyemids were truly primitive multicellular animals [2, 6, 13, 15, 23, 30]. Their general mode of life and life cycle are reminiscent of those of parasitic flatwarms. Stunkard held that dicyemids were degenerate flatworms [35]. In the life cycle of dicyemids, vermiforms develop from a single cell (agamete) asexually. Similar asexual reproduction is rare among other animals. The development of a redia within the miracidium in the life cycle of digenetic trematodes [31, 32] is analogous to the development of vermiforms. In terms of life cycle, the miracidium appears to correspond to the infusoriform; both are formed from fertilized eggs. In both groups of animals, dispersal larvae develop only from fertilized eggs. In view of such a similarity of thier life cycle, Stunkard [35], Bogolepova [3], and Ginetsinskaya [8] suggested the idea of phylogenetic affiliation between trematodes and dicyemids. There is certainly some resemblance between the infusoriform and the miracidium; both develop from a fertilized egg and are composed of a constant number of cells [14]. Cell constancy is further seen in all known stages of dicyemids, but in any stage of trematodes, except the miracidum, such a cell constancy is unknown. The cell constancy in dicyemids is strongly reminiscent of that of

aschelminths. McConnaughey [26] suggested that dicyemids may be related to very early progenitors of aschelminths or to certain of the earliest aschelminths. Programmed cell death has also been noted in the embryogenesis of the nematode Caenorhabditis elegans [36]. However, there is apparently no further evidence to support any relationship between dicyemids and aschelminths.

Spiral cleavage in dicyemids is reminiscent of that in flatworms (Platyhelminthes) and this similarity may be used again as an argument for a phylogenetic relationship between dicyemids and flatworms. In acoels, however, cleavages proceed by duets and small blastomeres are not produced at the vegetal pole [1]. In polyclads, early cleavages proceed by quartets as in dicyemids, but four small macromeres produced at the vegetal pole of the polyclad embryo are later absorbed during embryogenesis [17]. Details of developmental patterns, thus, are different between dicyemids and flatworms. In sponges (Porifera), the early cleavages are usually radial and , in coelenterates (Cnidaria), it is radial, bilateral, or partly spiral [37]. The cleavage patterns of these primitive invertebrates are so diverse that a similarity in cleavage pattern per se may not necessarily reflect a phylogenetic relationship between the organisms concerned. Development of placozoans, Trichoplax adhaerens, and orthonectides, Rhopalura ophiocomae, have been only partly described [5, 7, 19]. Detailed comparative studies on the development of these "mesozoan" animals are necessary if we are to gain any insight into details of the evolution of these animals. In orthonectids, a germ cell within ciliated larvae forms the plasmodium, in which agametes develop into either male

or female individuals [5, 18]. The life cycle of orthonectids is somehow comparable to that of dicyemids, but the body organization of orthonectids is considerably different from that of dicyemids [18, 19].

In embryogenesis of dicyemids, cell divisions appear to be highly determinate and result in an embryo with a definite number and arrangement of cells. Cell death and unequal divisions occur in a certain cell lineage during embryogenesis. Thus, the embryogenesis of dicyemids seems to be highly programmed. In infusorigens, the developmental process may be also determinate and programmed. The developmental pattern of dicyemids is very simple and little plasticity apparently exists in the development as discussed in a previous section. However, it remains still debatable whether these developmental "specializations" of dicyemids are solely attributed to the parasitic mode of life. A recent work using the morphological data base exhibits that the level of character loss in the parasitic platyhelminths is not unusually high [4]. Even if flatworms had lost their characters during the parasitic mode of life, the degree of the loss might not attain to the level of tissue. It may be difficult to understand that parasitic platyhelminths degenerate to the level of dicyemids which do not have tissues and are devoid of visible extracellular material.

Phylogenetic relationship of dicyemids has recently been studied by using modern methods of molecular biology, but the results are different in two molecule species used. Studies on sequences of 5S ribosomal RNA have suggested that dicyemids are somewhat closer to ciliate protozoans than to flatworms [30],

while as to the nucleotide sequence of 18S ribosomal DNA, dicyemids come into a group of triploblastic animals [16]. It is badly needed to study the nucleotide sequence of 28S ribosomal DNA of many primitive invertebrate taxa including dicyemids. However, different molecular approaches do not necessarily give a consistent result. More traditional methods such as comparative morphology and embryology are still indispensable to validate molecular results, if such methods are adequately applied and results are carefully interpreted.

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SUMMARY

1. Dicyemid mesozoans from the renal sacs of both Octopus vulgaris and Octopus minor was studied, and two new species that belong to the genus Dicyema were found. Dicyema japonicum sp. nov. from O. vulgaris, is a medium sized dicyemid, rarely exeeding 1500 μm in length. The disc-shaped calotte and parapolar cells form the cephalic enlargement. Dicyema clavatum sp. nov. is a relatively small sized dicyemid, infrequently reaching 1000 μm in the length, and this is the first mesozoan species described from O. minor. The calotte is cap-shaped and smoothly rounded. In addition, fifteen undescribed dicyemid species were detected from seven cephalopod species obtained of the coast of Japan.

2. The cleavage pattern and cell lineage of the infusoriform embryo of the D. japonicum were studied. The early cleavages are holoblastic and spiral. At around the 20- to 24-cell stage, cleavage becomes asynchronous and its pattern changes from spiral to bilateral. The four micromeres, namely, presumptive germinal cells, do not divide further and are finally incorporated into the cytoplasm of four urn cells, which are generated after divisions of the sub-macromeres. Two blastmeres ($2a^2$ and $2d^2$) undergo extremely unequall divisions and the much smaller sister blastmeres degenerate and ultimately disappear during embryogenesis. The fully formed embryo consists of 37 cells. The complete knowledge of cell lineage may provide the basis for further study on cell differentiation and morphogenesis.

3. The development of the functionally hermaphroditic gonad, the infusorigen, in four dicyemid species, D . orientale, D. acuticephalum, D. japonicum, and D. misakiense, was studied. After an agamete (axoblast) undergoes the first division and excludes a paranucleus, the resulting cell undergoes the second division. Afterwards, three different types of cell lineage can be identified. In all species examined, oogonia occupy the outer surface of the axial cell of the infusorigen and spermatogonia are incorporated into the axial cell of the infusorigen. In this way, the spermatogenesis proceeds within the cytoplasm of the axial cell. The difference of cell lineage of the infusorigen could be used as one of criteria for classification of dicyemids.

4. The pattern of cell division and cell lineage of the vermiform embryos of dicyemids were studied using specimens of two species, namely, D. acuticephalum, which has 16 to 18 peripheral cells, and D. japonicum, which has 22 peripheral cells. The division proceeds spirally in the early stage but becomes bilateral from the fifth cell division onward. In two lines of cells, namely, the line descended from the prospective axial cell and the line from the mother cell of the head, the programmed cell death occured. Differences in the number of peripheral cell are due to the number of times that divisions of the mother cells occur. The cell lineage of the calotte differs between D. acuticephalum and D. japonicum.

5. Survival times of nematogens and infusoriform larvae *in vitro* in the octopus urine and in seawater were studied. Nematogens have been alive more than for ten days in the urine, but they died within five days in seawater. The infusoriform larvae became immobile only within two days in both the urine and seawater. Dicyemids were also cultured *in vitro* in an artificial medium. They could be kept for over one month. During this *in vitro* culture, two kinds of embryos, vermiform embryos and infusoriform embryos, developed within the axial cells of nematogens and rhombogens, and they escaped into the medium apparently in a healthy condition.

6. In embryogenesis of dicyemids, cell divisions appear to be highly determinate and results in an embryo with a definite number and arrangement of cells. The embryogenesis of infusoriform embryos and vermiform embryos may be highly programmed. Thus, cell death and unequal divisions occur in a certain cell lineage during embryogenesis. On the whole, the developmental pattern of dicyemids is very simple and little plasticity apparently exists in the development. The dicyemids may be not only a simple primitive organism, but also a specialized organism.

ACKNOWLEDGMENTS

I would like to express my deep and heartfelt gratitude first to Dr. Yutaka Koshida, the Professor Emeritus of the Faculty of General Education of Osaka University, who kindly introduced me to the biology of dicyemid mesozoans and guided me during the master and doctor courses. My hearty gratitude is also due to Dr. Kazuhiko Tsuneki, for his valuable advice, support, and encouragement. I also would like to express my sincere thanks to Dr. Toshiteru Morita, Dr. Yasuo Nakanishi, Dr. Kazuo Ito, and Dr. Shinri Horiuchi for their supports and encouragements during the course of this work, and to Ms. Kikuko Kurihara, Ms. Yoko Saegusa, and my wife, Akiko, for their valuable assistance during the preparation of this manuscript.