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ULTRASTRUCTURAL FEATURES OF THE CYTOPLASM OF RESPECTIVE KINDS OF CELL LINEAGES OF CLEAVAGE-ARRESTED ASCIDIAN EMBRYO

BY

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ABSTRACT

We observed the ultrastructual features of the cytoplasm in the cell lineages of the cleavage-arrested embryos of the ascidian, *Halocynthia roretzi*, and compared them with those of the cytoplasm in the corresponding cell lineages of the normal embryos or in the corresponding tissues of the normal larva, in order to examine the presence or the degree of the cellular differentiation without the cleavage.

The cleavage of the embryos was arrested by the cytochalasin B at the 16-cell stage and the embryos were cultured in sea water containing cytochalasin B until the developmental time equivalent to the hatching of the control larva.

As a result, the cytoplasmic components in the cleavage-arrested 16-cell embryos resembled those observed in the corresponding tissues of the normal larvae, especially in the b5.3 and b5.4 blastomers (epidermal lineage cells) and in the B5.1 blastomeres (which give rise to the endodermal, mesenchymal and muscle cells) but they were less uniformly organized and their polarity or orientation was very random and irregular, compared with those in the normal larvae.

These results demonstrated that the morphological differentiation similar to the larval tissue differentiation could proceed even in the cleavage-arrested embryos to a certain extent.

Key words: Ascidian embryo, Larva, Cytochalasin B, Cleavage-arrest, Cell-lineage, Golgi apparatus, Secretory granules, Myofilaments, Cellular differntiation, Egg localization

INTRODUCTION

In the ascidian embryos, regional cytoplasmic differences appear immediately after fertilization via the transient cytoplasmic movement called ooplasmic segregation (Conklin [1]). The developmental fate of each region is clearly restricted (Conklin [1, 2]; Ortolani [3–5]; Reverberi [6]). Our previous study [7] demonstrated that the embryonic regions or cells, which differentiate into specific types of larval tissues later, have a characteristic distribution mode of the organelles in the cytoplasm. Because the developmental fate of the embryonic cell appears to be specified according to the sectional region of the embryonic cytoplasm inherited by it, it has been considered that the cytoplasmic determinants are localized in each region and that those determinative agents are segregated by a determinate cleavage pattern into certain cell lineages where they

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appear to play a role in programming the differentiation pathways of the cell (Wilson [8]; Davidson [9]). Therefore, the analysis of the differentiation in the cleavage-arrested embryo is thought to afford an opportunity to investigate the existence of the cytoplasmic factors and the mechanisms whereby the cytoplasmic factors become segregated within the developing embryo. Lillie's observation [10] has been famous, as an example, indicating the differentiation without the cleavage. Whittaker [11–13] took advantage of this observation of Lillie's and extended his discovery to the demonstration that two tissue-specific enzymes,— muscle acetycholinesterase and brain pigment cell tyrosinase— are able to develop in certain blastomeres of the ascidian eggs and embryos whose cleavage was arrested by cytochalasin B. These blastomeres match the known cell lineage patterns for the two enzyme-containing tissues. Recently it has been reported that membrane differentiation, such as electrical excitability, occurs in the cells of the ascidian embryo whose cleavage is arrested by cytochalasin B at various stages during the early development and cultured for a certain period afterwards, (Takahashi and Yoshii [14]). In this paper, we investigated the presence or the degree of the differentiation in the embryonic cell lineages of the cleavage-arrested ascidian embryos from the morphological point of view. The results demonstrated that the morphological differentiation similar to the larval tissue differentiation could proceed even in the cleavage-arrested embryo, although the polarity or orientation of the cytoplasmic components characteristic of the larval tissues was random or irregular in the cleavage-arrested embryo. These findings lend to a certain degree support to the theory that the localized and segregated egg cytoplasmic determinants are responsible for the larval tissue development in the ascidian embryos. However, considering that the blastomeres in the cleavage-arrested 16-cell embryo did not clearly develop mosaic ultrastructures but differentiated into one or at most two types of larval tissues even if they have more than two potential fates, it is suggested that the detailed mechanism of differentiation in the cleavage-arrested embryo should be further clarified.

Materials and Methods

Materials

The eggs and sperms of the ascidians (*Halocynthia roretzi*) were kindly offered to us by Prof. Kunitaro Takahashi and Dr. Tomoo Hirano who reared the spawning ascidians through late autumn and winter (Hirano and Takahashi [15]; Hirano *et al.* [16]). Fertilization was carried out by mixing the eggs and sperms taken from another animal together. The fertilized eggs were cultured in the Petri dishes placed on a slow-moving table in a water bath kept at a constant temperature of 7–9°C. The morphological stages of the embryo were determined according to the description by Conklin [13], the previous study on this species by Ohmori and Sasaki [17] and also according to the scanning electron microscopical (SEM) observations by Satoh [18, 19]. At the 16-cell stage, the developing embryos were transferred to another Petri dish containing 1–2 μg/m*l* cytochalasin B (Aldrich) in sea water, a concentration sufficient to block cytokinesis (Takahashi and Yoshii [14]). The cleavage-arrested embryos were cultured until the control tadpole larvae hatched.

Specimen preparation, light and electron microscopy, identification of blastomeres and mapping of organelle region

See the previous paper (Nishimura

and Wake [7]

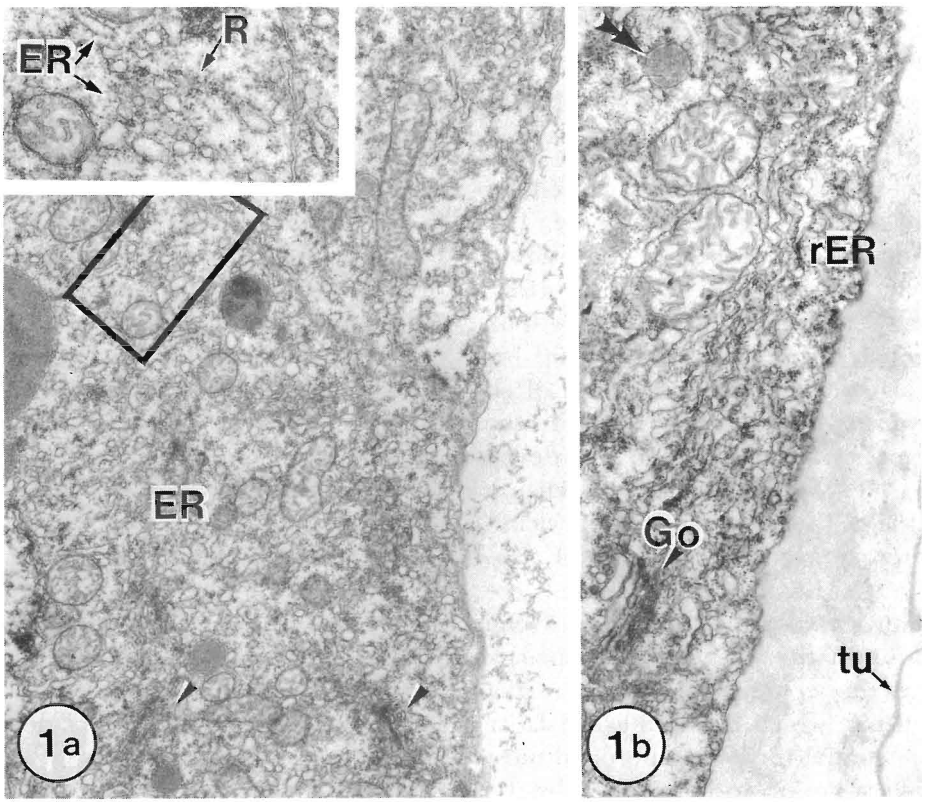
Acetylcholinesterase staining

The cleavage-arrested embryos and larvae were fixed in 10% formalin in sea water for 30 minutes to 1 hours at 4°C and stained for acetylcholinesterase (AchE) by the 'direct-colouring' thiocholine method (Karnovsky and Roots [20]). The details of this staining procedure are the same as described by Ohmori and Sasaki [17] and Takahashi and Yoshii [14]. The embryos and larvae were then washed with distilled water, dehydrated through a graded series of ethanol and cleared with propylene oxide, in which the chorion and follicular envelope of the embryo were removed manually using fine needles under a binocular microscope. The embryos and larvae were embedded in epoxy resin. Two semithin sections (0.5–1 μ) were cut with glass knives at intervals of 5–8 μ. The first ones were mounted on the slides, enclosed in Canadian balsam immdediately and examined with a conventional microscope. The second ones were also mounted on the slides, stained with 0.5% toluidine blue and examined with the same microscope.

Results

I. *Ultrastructures in the neural, epidermal and muscle cells of normal larvae*

Normal embryos which continued its development at 9°C without being treated with cytochalasin B for the experimental control begin to hatch in about 68 hours into a free-swimming tadpole larvae consisting of a trunk and a tail region. As previously reported (Nishimura and Wake [7]), the larval tissues respectively had a characteristic distribution



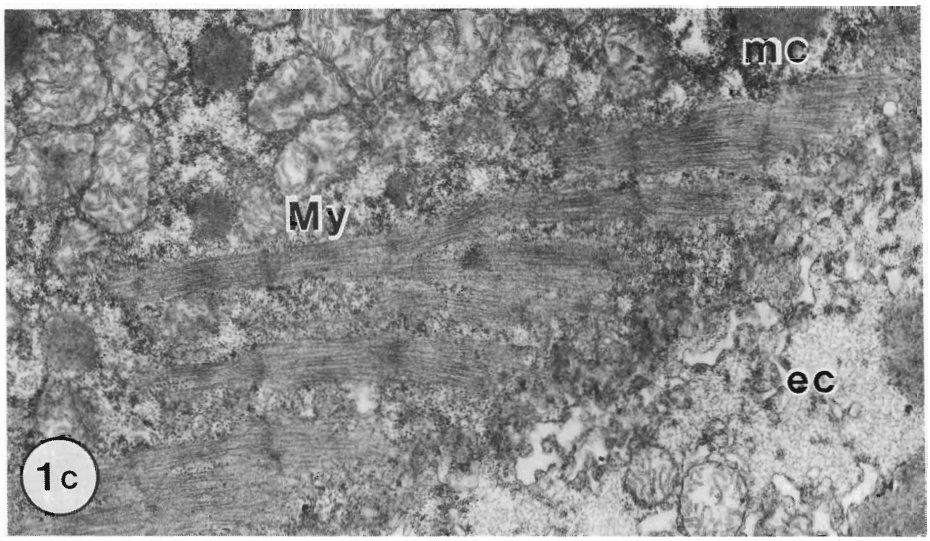


Fig. 1. Ultrastructural features in larval tissues.
(a): Neural cell. ER, mainly ER vesicles to which ribosomes are often attached and free ribosomes (R) are closely packed in the cytoplasm (×6100). Insert shows a higher magnification of ER vesicles (×12900). Arrowheads: Golgi apparatus.
(b): Epidermal cell. Well-developed Golgi apparatuses (Go) are lined up along the cell surface and a secretory granule (double arrowhead) is visible at the top of the micrograph. Rough ER (rER) whose cisternae are abundant are found near the Golgi apparatus in the peripheral cytoplasm (×10300).
tu: Tunic.
(c): Muscle cell (mc). Myofibrils (My) are arranged beneath the sarcolemma. Numerous mitochondria are found in the medulla of the cytoplasm (×9300).
ec: Epidermal cell.

mode of the organelles, which turned out to exist in the 8-cell stage already. The entire neural cells in the larva were abundant in endoplasmic reticulum (ER), especially rough ER (rER), which took the tubular or vesicular form. And besides, a few Golgi apparatus were often found in the neural cell (Fig. 1a). In the epidermal cells, a number of well-developed Golgi apparatus were lined up along the cell surface and in their vicinity, secretory granules were found (Fig. 1b). Rough ER whose cisternae were abundant was also found near the Golgi apparatus in the peripheral cytoplasm. Outside the epidermis, there was the larval tunic, which is a protective layer and is essential for locomotion and most of which is known to be secreted by the epidermis (Deck *et al.* [21]; Terakado [22]). Moreover in the larval muscle cells, we confirmed the orderly lined up myofibrils beneath the sarcolemma (Fig. 1c). The length of the A-band was about 1.4 μ, which was almost equal to that of the skeletal muscle. But the sarcoplasmic reticulum (SR) and T-system were poorly developed.

II. *Ultrastructures in respective cell lineages of 16-cell embryos*

A. Location of all blastomeres in 16-cell embryo and their developmental fate

The cytochalasin-arrested 16-cell embryo remained with morphological features similar to those at the beginning of the drug application, that is, to those in the normal 16-cell embryo, though the

former had some vacuoles between the blastomeres. Therefore, as to the location of the blastomeres, the cleavage-arrested and the normal 16-cell embryo were the same (Figs. 2 and 3). The location of all the blastomeres in the 16-cell embryo and their developmental fate given by Conklin [1], Ortolani [3–5], and Reverberi [6] are as follows; In the 16-cell embryo, eight relatively small blastomeres, pairs of a5.3, a5.4, b5.3 and b5.4 blastomeres constitute the animal hemisphere, as shown in *Figs. 2a and 3a*. Two pairs of blastomeres (the a5.3 and a5.4 blastomeres) located anteriorly will give rise to both the neural and epidermal cells. Two pairs of small blastomeres (the b5.3 and b5.4 blastomeres) located posteriorly in the animal hemisphere are exclusively presumptive epidermal cells. *Figs. 2c, 2d and 3b* show the eight large blastomeres in the vegetal hemisphere of the 16-cell embryo. The four round cells lined up anteriorly are two pairs of blastomeres (the A5.1 and A5.2 blastomeres) which will give rise to the endodermal, notochordal and neural cells. The two large blastomeres in the middle zone of the vegetal hemisphere are the B5.1 blastomeres which will give rise to the endodermal, mesenchymal and muscle cells. Finally, the pair of small blastomeres at the posterior end of the vegetal hemisphere are the two B5.2 blastomeres, which will give rise to the muscle and mesenchymal cells.

B. Ultrastructures in respective cell lineages of cleavage-arrested 16-cell and normal 16-cell embryos

1. a5.3 and a5.4 blastomeres—neural and epidermal precursor cells

In the cleavage-arrested 16-cell embryo, both the a5.3 and a5.4 blastomeres were rich in ER throughout the cytoplasm. *Fig.4* shows an example of the fine structure in the a5.4 blastomeres. They have a small number of yolk granules in the peripheral cytoplasm. The Golgi apparatus assuming a whirling form was also observed at the periphery but the secretory granules were poorly developed (Figs. 4a and 4b). Many residual bodies were observed in the interior

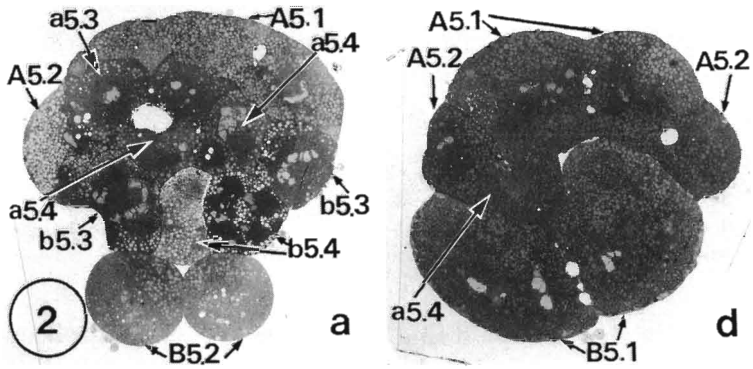


Fig. 2. Light micrographs of sections of cleavage-arrested 16-cell embryo.
(a)–(d): Sections cut vertical to the animal-vegetal axis from the animal hemisphere to the vegetal hemisphere. (a)–(c)×100, (d)×120.
(e): Higher magnification of the b5.3 blastomere showing a large number of black string-like structures (*) (×210).
(f): Higher magnification of the B5.1 blastomere showing the yolk mass in the large central part of the cytoplasm and faint filamentous structures in the scanty peripheral cytoplasm (×210).
The anterior end is up and the posterior end down in all figures.

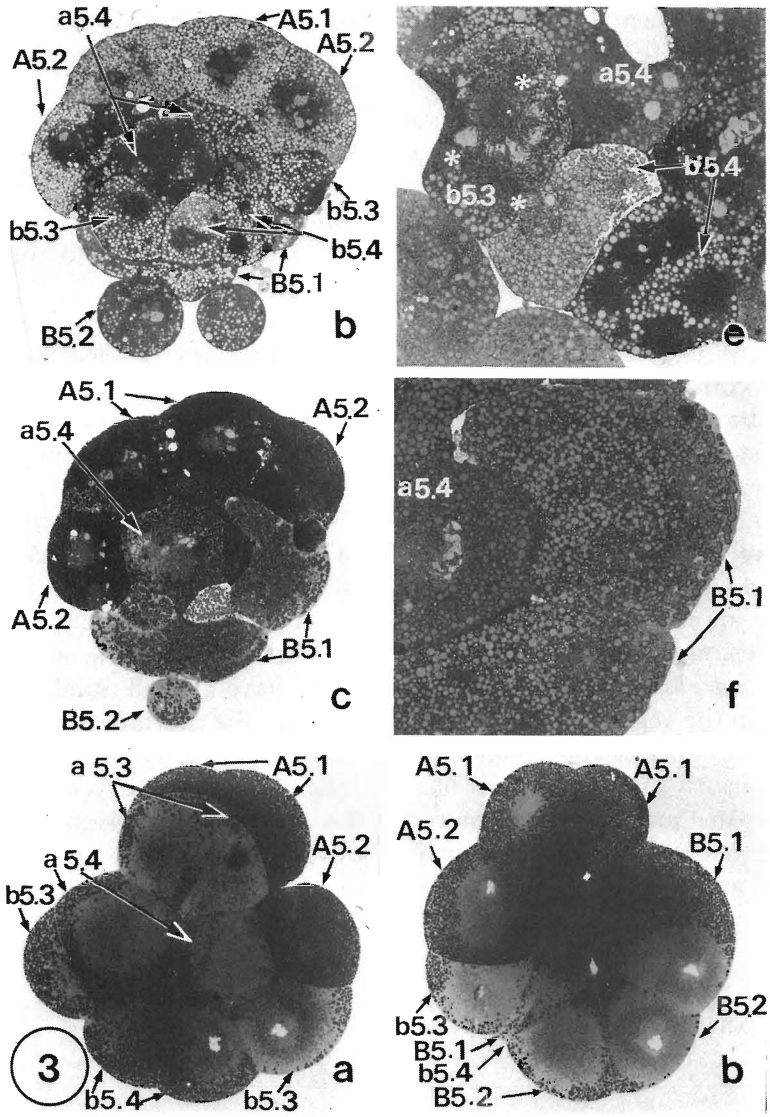


Fig. 3. Light micrographs of sections of normal 16-cell embryo.
(a): Section cut vertical to the animal-vegetal axis in the animal hemisphere (×120).
(b): Section cut vertical to the animal-vegetal axis in the vegetal hemisphere (×110).
The anterior end is up and the posterior end down in both figures.

of the cytoplasm (Fig. 4c). We could find the annulate lamellae surrounded by many ER, especially the vesicular rER, in the cytoplasm on the A5.2 side of the a5.4 blastomere (Fig. 4d). In the normal 16-cell embryo, both the a5.3 and a5.4 blastomeres were very abundant in ER throughout the cytoplasm, as in the cleavage-arrested 16-cell embryo (Fig. 5), however, neither the Golgi apparatus nor the residual bodies were present in the normal 16-cell embryo.

2. b5.3 and b5.4 blastomeres—epidermal precursor cells

Comparatively many yolk granules were found both in the b5.3 and b5.4

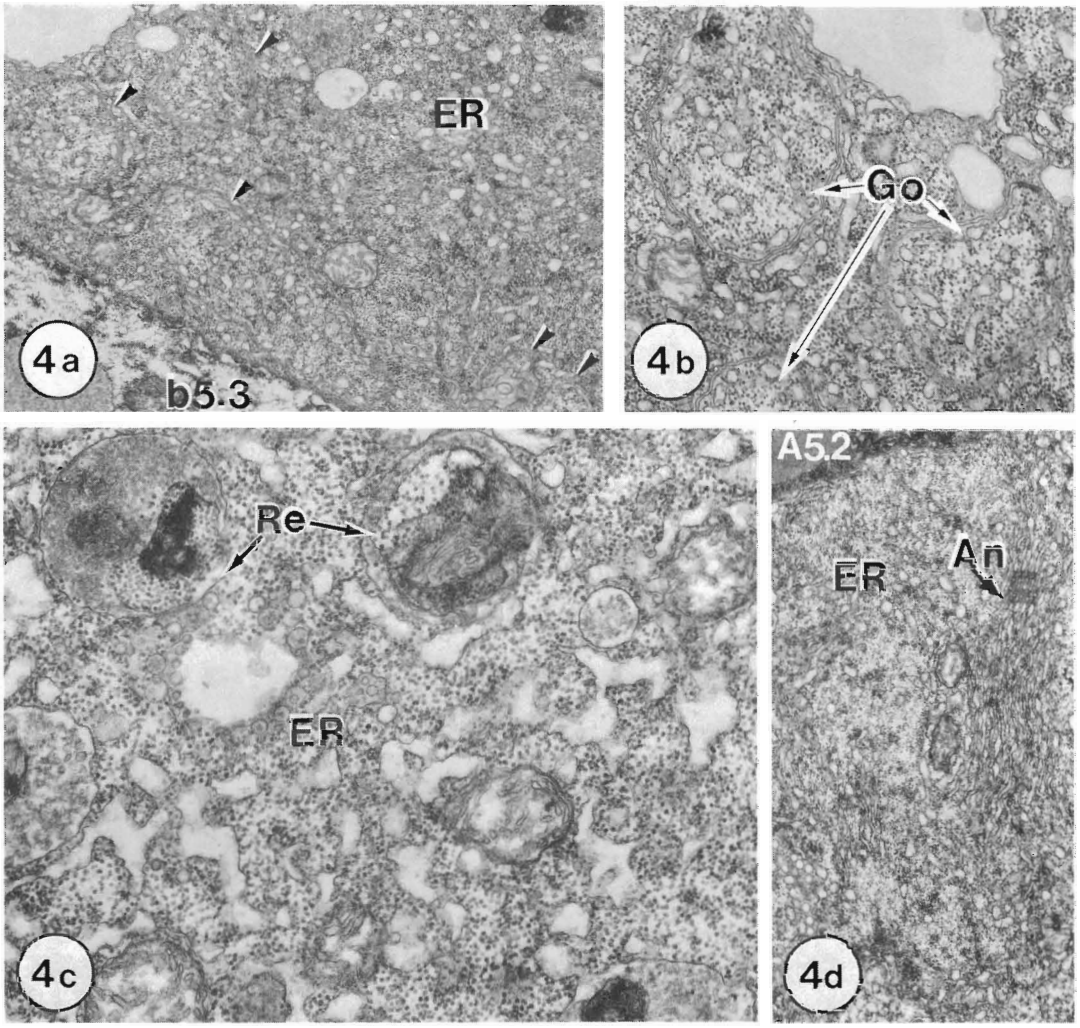


Fig. 4. Ultrastructure of a5.4 blastomeres in cleavage-arrested 16-cell embryo.
(a): Peripheral cytoplasm in the a5.4 blastomere. ER is closely packed and a number of Golgi apparatuses (arrowheads) which assume a whirling form are found.
At the bottom of the micrograph, the b5.3 blastomere is found (×5900).
(b): Higher magnification of whirling Golgi apparatus (Go) (×10500).
(c): Interior cytoplasm in the a5.4 blastomere. ER is very abundant and residual bodies (R) are found (×1700).
(d): Annulate lamellase (An) surrounded by abundant ER. We happened to be able to find the annulate lamellae in the cytoplasm on the A5.2 side of the a5.4 blastomere (×6400).

blastomeres of the cleavage-arrested 16-cell embryo (Figs. 2a and 2b). In many parts of their cytoplasm, there were many Golgi apparatus which appeared as black string-like structures with light microscopy (Fig. 2e) and great many secretory granules. These Golgi apparatus took a large ring formation but their polarity was random (Fig.6). Moreover, the rER abundant in the cisternae, which is usually present in the secretory cells, was observed adjacent to the cytoplasm where numerous Golgi apparatus and secretory granules were distributed (Fig.

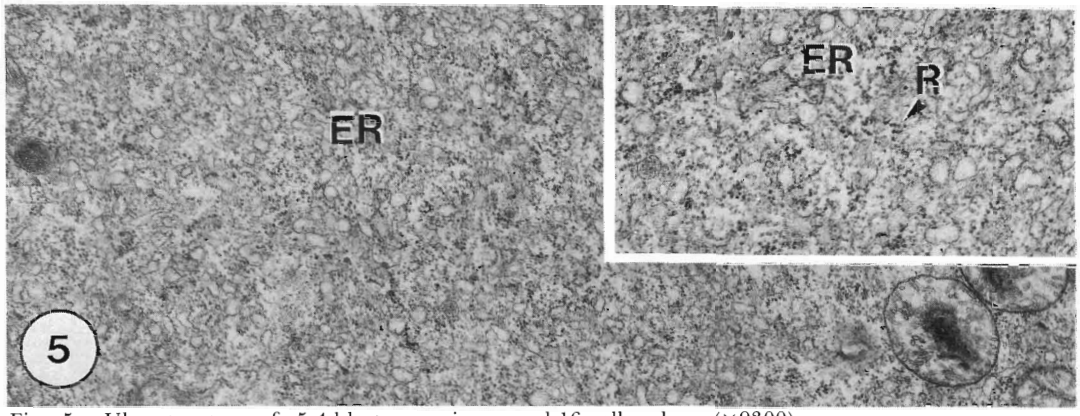
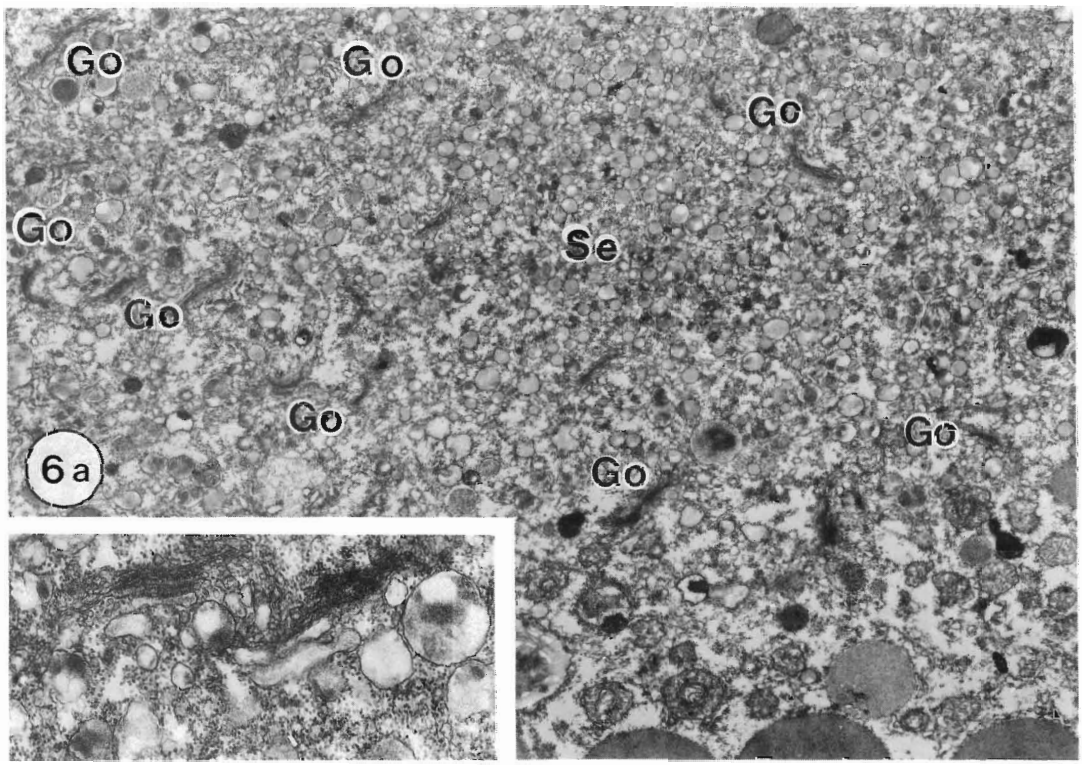


Fig. 5. Ultrastructure of a5.4 blastomere in normal 16-cell embryo (×9300).
ER and *ribosomes* (*R*) are very abundant in the cytoplasm. Insert shows a higher magnification of ER vesicles and ribosomes (R) (×13400).



6b). These findings implied the expression of the secretory function in the b5.3 and b5.4 blastomeres of the cleavage-arrested embryo. This function characteristic of the larval epidermal cells as shown on *Fig. 1b*.

However, the formation of the tunic, known to be mainly secreted by the epidermal cell (Deck *et al.* [21]; Terakado [22]), was not clearly observed. This was probably due to the inhibition of the secretion by cytochalasin B (Williams and

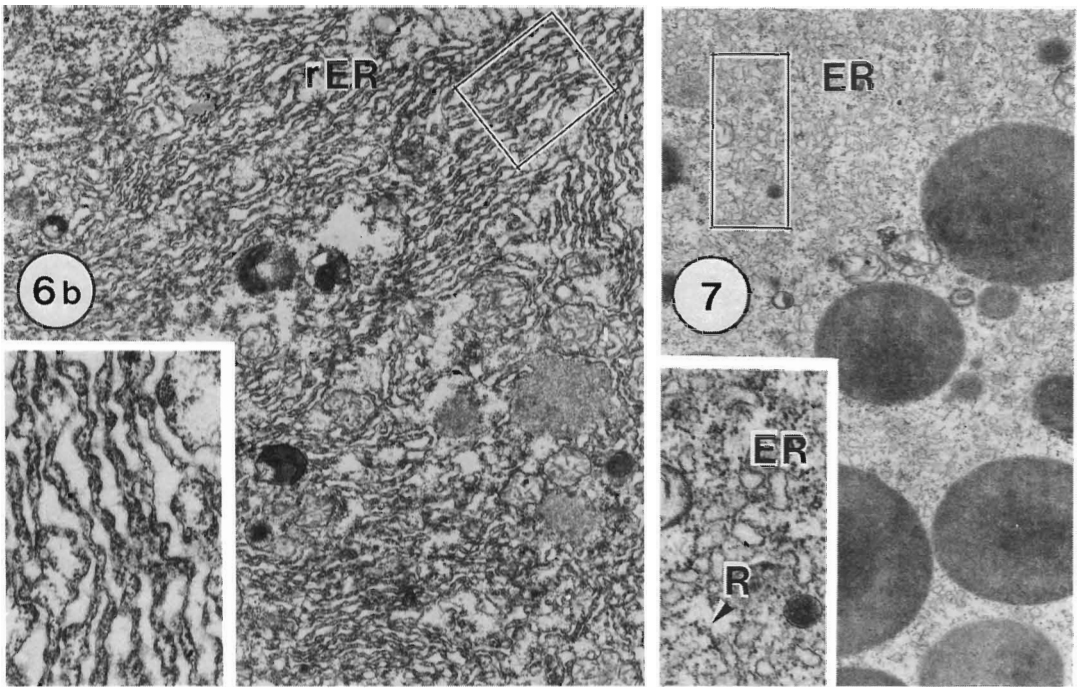


Fig. 6. Ultrastructure of b5.3 blastomeres in cleavage-arrested 16-cell embryo.
(a): The region abundant in Golgi apparatus (Go) and secretory granules (Se), (×4700). Insert shows Golgi apparatus (×13500).
(b): The region abundant in rER. This region is adjacent to the region in (a) (×5100). Insert shows higher magnification of cisternae o rER (×14100).

Fig. 7. Ultrastructure of b5.3 blastomere in normal 16-cell embryo.
The ER and ribosomes are conspicuous and besides a comparatively large number of yolk granules are found (×5400). Insert shows the vesicles of ER and ribosomes (R) (×12200).

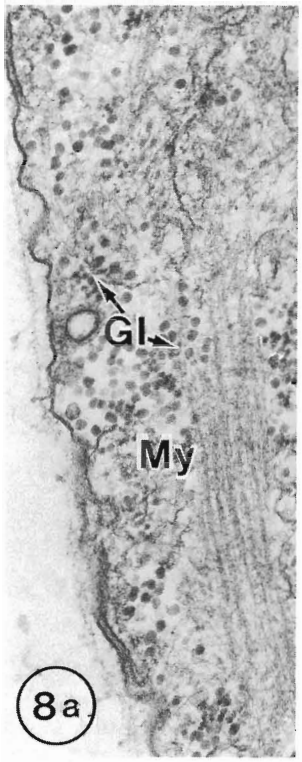
Wolff [23]). The b5.3 and b5.4 blastomeres in the normal 16-cell embryo had abundant vesicular ER and a little more yolk granules than the a5.3 and a5.4 blastomeres (Fig. 7). The Golgi apparatus was not present, but there were much more secretory granules.

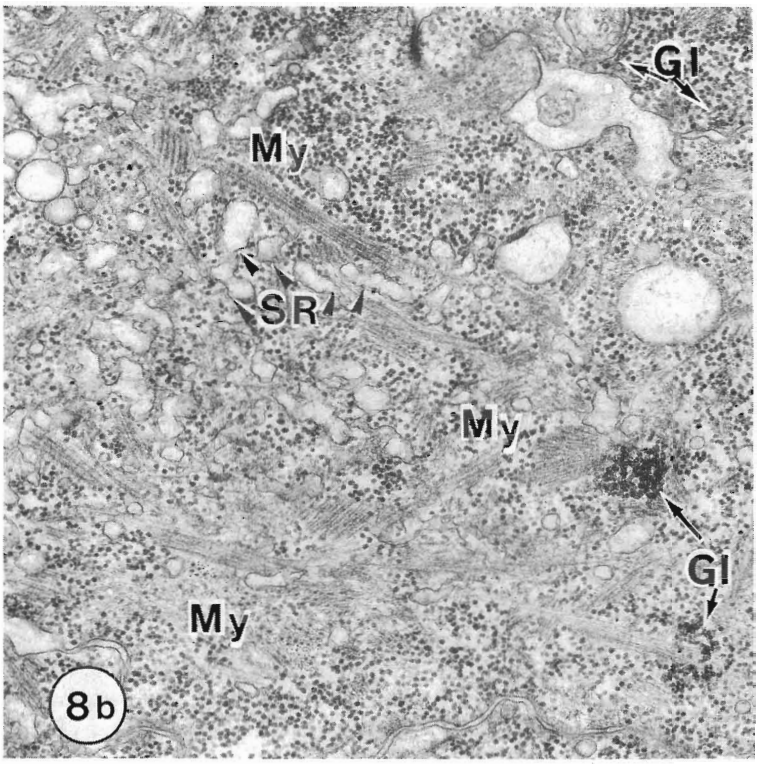
3. A5.1 and A5.2 blastomeres—endodermal, notochordal and neural precursor cells

A large number of yolk granules was closely packed throughout the cytoplasm in the A5.1 and A5.2 blastomeres of the cleavage-arrested 16-cell embryo (Figs. 2a to 2d). But the vacuolation, which was observed in the larval notochordal cells as previously reported (Nishimura and Wake [7]), was indistinct in these blastomeres of the cleavage-arrested embryo, although they had the potentiality to become the notochord as one of their potential fate. The A5.1 and the A5.2 blastomeres in the normal 16-cell embryo were very abundant in the yolk granules and ribosomes (Figs. 3a and 3b). The vacuolation did not occur in these blastomeres yet.

4. B5.1 blastomeres—endodermal, mesenchymal and muscle precursor cells

Many yolk granules occupied a large part of the interior cytoplasm of the B5.1 blastomeres in the cleavage-arrested 16-cell embryo (Figs. 2c and 2d). These


Gl
My
8a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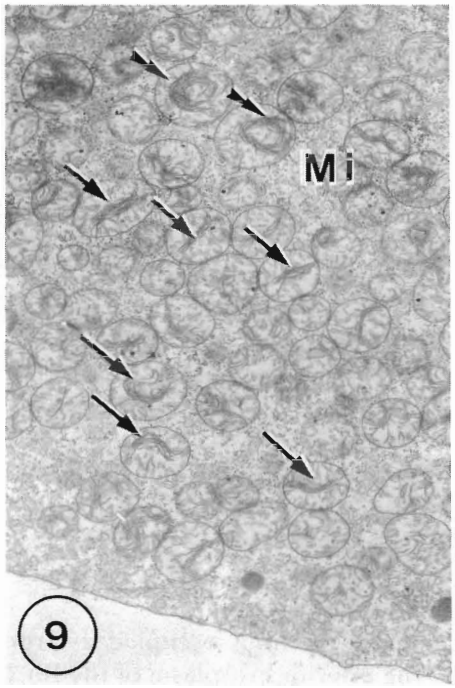
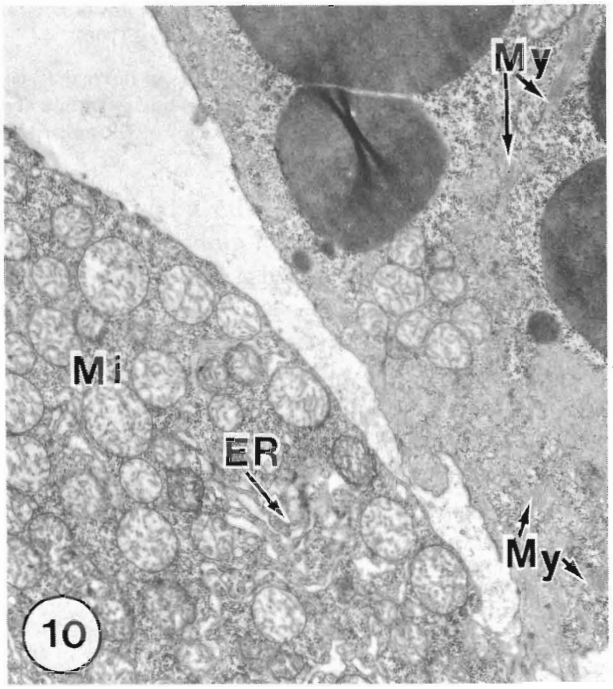

Mi
9


My
Mi
ER
My
10

blastomeres had a number of very small aggregates of mitochondria mainly at the periphery in their cytoplasm. Many bundles of myofilaments consisting of thick and thin filaments which were arranged in parallel, and to a lesser extent the individual myofilaments were dispersed among the mitochondria, mainly in the small peripheral cytoplasm of these cleavage-arrested blastomeres (Figs. 8a and 8b). These parallel arrays of myofilaments resembled those of the normal larval muscle in thickness and density (Fig. 1c). But they were less uniformly organized and, furthermore, they were randomly oriented (Fig. 8b). In the bundle the structure corresponding to the Z-line was not observed clearly. In addition, a well-developed sarcoplasmic reticulum (SR) was present in the peripheral cytoplasm abundant in the bundles of myofilaments, but few membranes of SR were found associated with the myofilaments. Numerous glycogen granules were also distributed densely in the myofilament-rich regions of the cytoplasm. On the other hand, the B5.1 blastomeres in the normal 16-cell embryo had large aggregates of mitochondria in the extensive region of the cytoplasm (Fig. 9). Mitochondrial cristae were more complex in the normal 16-cell embryo than in the cleavage-arrested 16-cell embryo. However, they did not exhibit any muscle elements, of course.

5. B5.2 blastomeres—muscle and mesenchymal precursor cells

The B5.2 blastomeres, which, like the B5.1 blastomeres, have the potential to form the muscle (Conklin [1]; Ortolani [3–5]; Reverberi [6], did not synthesize any recognizable myofilaments in the cleavage-arrested 16-cell embryo (Fig. 10). Instead, the mitochondria and ER were mixed in almost the same proportion (~50%) in the large part of the cytoplasm. On the other hand, the B5.2 blastomeres in the normal 16-cell embryo were extremely rich in mitochondria like the B5.1 blastomeres in the normal 16-cell embryo.

III. *Comparison of ultrastructural features among the cleavage-arrested 16-cell embryo, the normal 16-cell embryo and the normal larva*

A. Mapping of organelle region in cleavage-arrested embryo

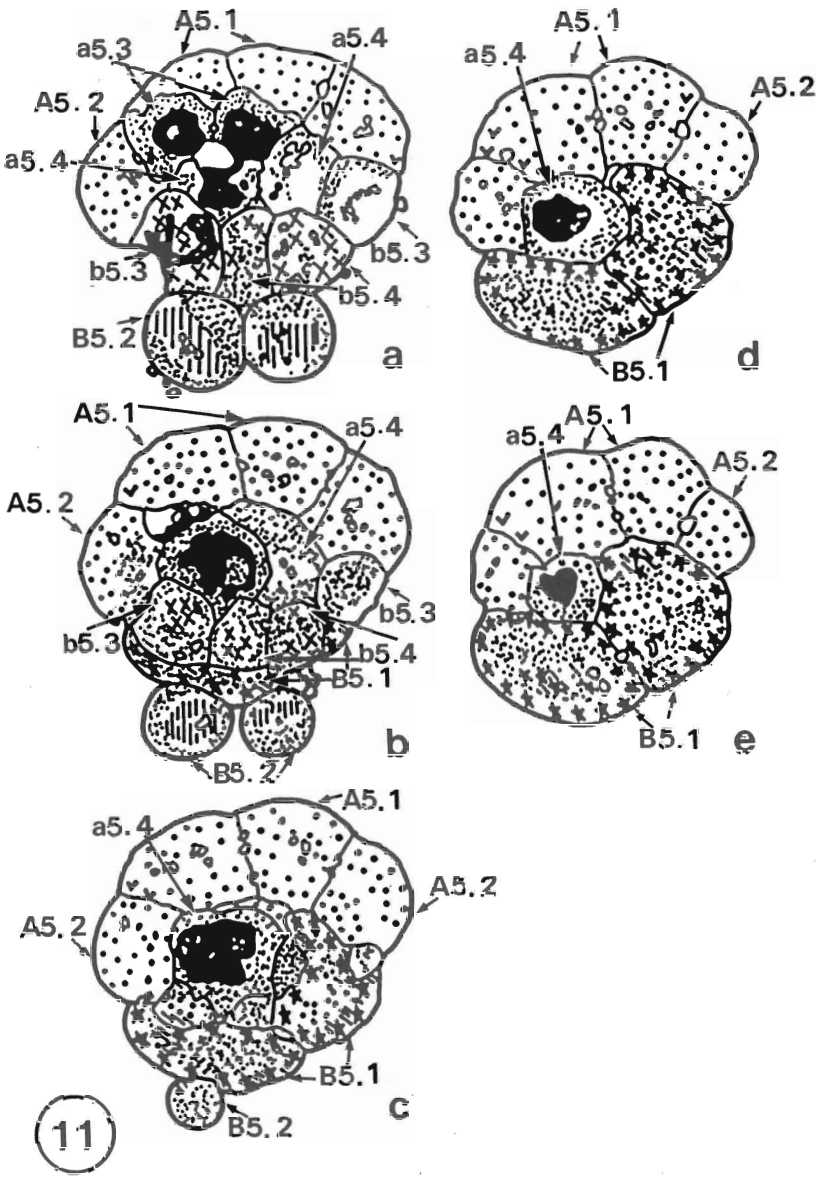
According to the observations of the ultrastructure, we examined the distribution density of the organelles in the

Fig. 8. Ultrastructure of B5.1 blastomers in cleavage-arrested 16-cell embryo.
(a): Parallel array of myofilaments (My) beneath the cell surface of the B5.1 blastomere (×4500).
Gl: Glycogen granules.
(b): The peripheral cytoplasm where parallel arrays of myofilaments (My) and to a lesser extent individual myofilaments are dispersed. Well-developed sarcoplasmic reticulum (SR) is found, but few membranes of the SR seem to be associated with the myofilaments (×25000).
Gl: Glycogen granules.

Fig. 9. Ultrastructure of B5.1 blastomere in normal 16-cell embryo Numerous mitochondria (Mi) fill the cytoplasm. Mitochondrial cristae are characteristic, that is, stratified cristae (arrows) or more complex and developed form of whirling cristae (double arrowheads) are often found in the center of mitochondria (×5000).

Fig. 10. Comparison of ultrastructures between B5.1 (right upside) and B5.2 (left downside) blastomere in cleavage-arrested 16-cell embryo.
The B5.2 blastomere does not seem to have any recognizable myofilaments (My) throughout the cytoplasm. Instead ER and mitochondria (Mi) are mixed in almost the same proportion (~50%) in the large part of the cytoplasm (×5100).

cleavage-arrested cells in the semiserial sections. Then some "organelle regions" were introduced in the cytoplasm on the section based on the distribution density of the organelles (see "Materials and Methods") and mapped in the traces of cleavage-arrested cells on the section with some symbols. *Fig. 11* shows the mapped cell traces on some sections from the animal hemisphere to the vegetal hemisphere in the cleavage-arrested 16-cell embryo. The Golgi region in the b5.3 and b5.4 blastomeres and the myofilament-dispersed region in the B5.1 blastomeres were recognized clearly in the cleavage-arrested embryo. Regional cytoplasmic differences were obscure and quite a large number of yolk granules



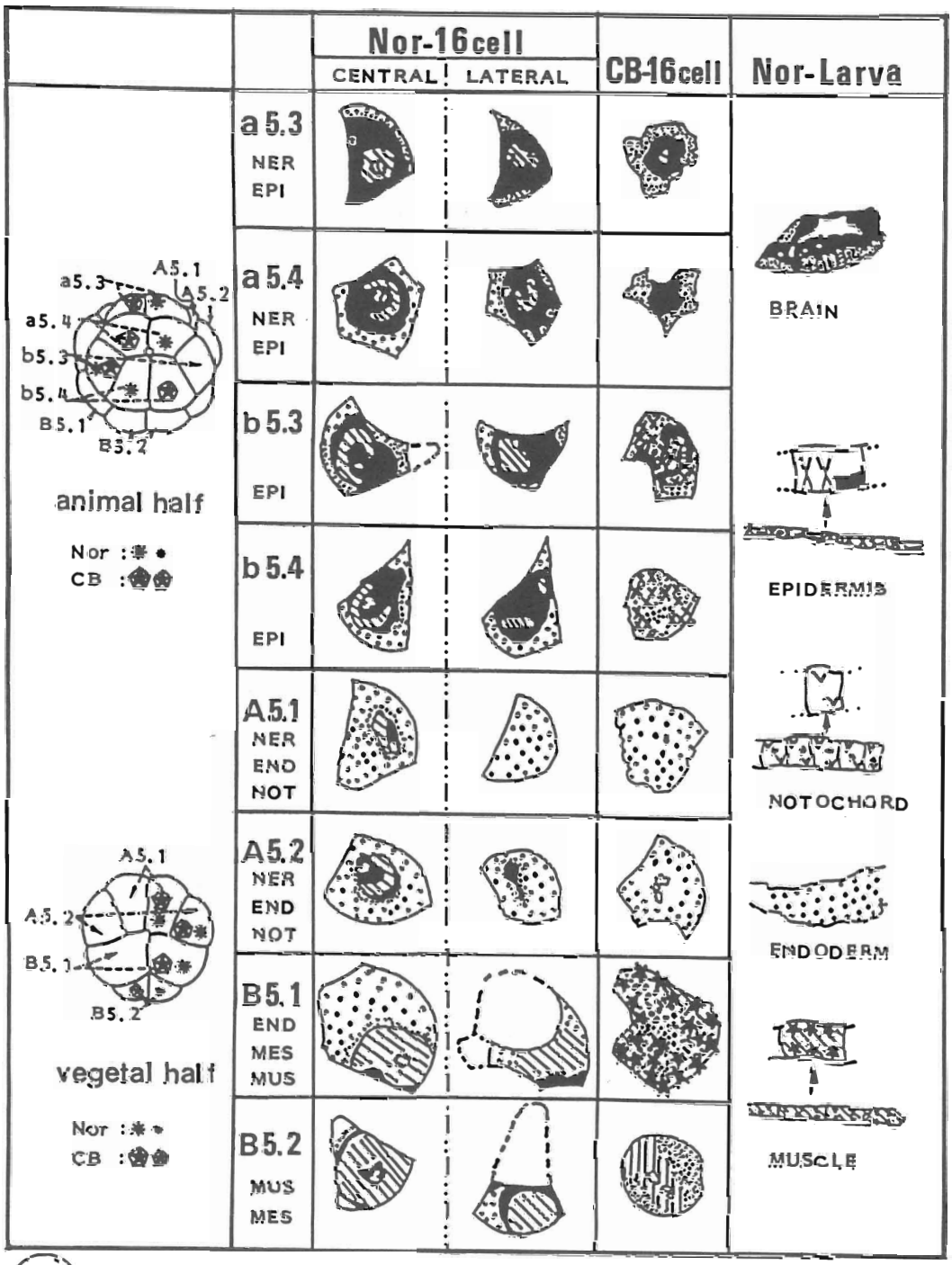
remained in the entire embryo.

B. Comparison of distribution mode of organelles in cytoplasm among cleavage-arrested 16-cell embryo, normal 16-cell embryo and normal larva

We also mapped the organelle regions in the normal 16-cell embryo and the normal larva, comparing the distribution mode of the organelles among the cleavage-arrested 16-cell embryo, the normal 16-cell embryo and the normal larva. The Golgi region and ER region were the most conspicuous regions in the animal hemisphere of the cleavage-arrested 16-cell embryo (Fig. 12), especially the Golgi region was peculiar to the b5.3 and b5.4 blastomeres which are exclusively presumptive epidermal cells. This region was also characteristic of the larval epidermal tissue, but, needless to say, this was not found in the b5.3 and b5.4 blastomeres of the normal 16-cell embryo. Adjacent to the Golgi region, the extensive ER region was found in these blastomeres of the cleavage-arrested embryo (Figs. 11 and 12). This ER region was mainly made up of the rER whose cisternae were abundant (Fig. 6b). Such a spatial relationship between the Golgi region and ER region is usually observed in what is called the secretory cells. Naturally this spatial relationship was observed in the epidermal cells of the ascidian larva (Fig. 1b). Therefore, the fine structures of the b5.3 and b5.4 blastomeres in the cleavage-arrested ascidian embryo were fairly analogous to those of the epidermal cells in its larva. But there were at least two differences in the fine structure between the b5.3 or b5.4 blastomeres of the cleavage-arrested embryo and the larval epidermal cells. One was that the Golgi region in the former was situated rather in the interior cytoplasm than in the peripheral cytoplasm along the cell surface (Figs. 11 and 12) and the other was that the polarity of the Golgi apparatus in the former was irregular whereas that in the latter was uniform (Fig. 6, 1b). On the other hand, the ER region was also found in the a5.3 and a5.4 blastomeres in the cleavage-arrested embryos. In these blastomeres, the ER region occupied most of the cytoplasm, which was also the characteristic feature both in the neural cells of the normal larva and in the a5.3 or the a5.4 blastomeres of the normal 16-cell embryo. But taking into account the appearance of a number of Golgi apparatus and residual bodies in the a5.3 and a5.4 blastomeres in the cleavage-arrested embryo, these blastomeres in the cleavage-arrested embryo seemed to resemble more closely the larval neural cells than the a5.3 and

Fig. 11. Mapping of organelle regions in cleavage-arrested 16-cell embryo (a)–(e).
Sections cut vertical to the animal-vegetal axis are indicated from the animal hemisphere to the vegetal hemisphere.

: ER region
: Mitochondrial region
: Yolk region
: Golgi region
: Vacuolated yolk region
: Subyolk region
: ER and mitochondria-mix region
: Myofibrils or myofilaments
: The region where the distribution density of the organelles did not apply to the criteria or where the organelle distribution could not be examined.


Nor-16cell
CENTRAL
LATERAL
CB-16cell
Nor-Larva
a5.3
NER
EPI
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EPI
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b5.4
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MUSCLE

12

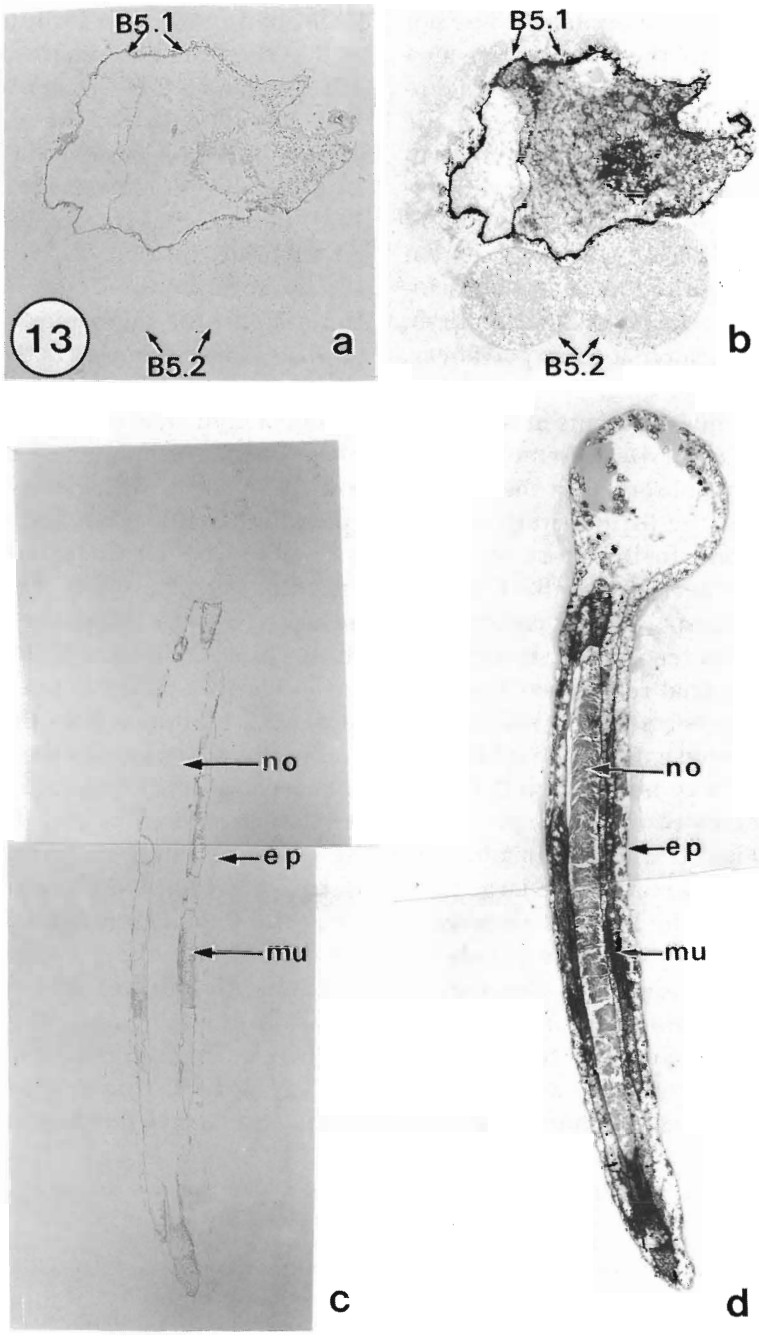
a5.4 blastomeres in the normal 16-cell embryo. Although in the 16-cell embryo both the a5.3 and a5.4 blastomeres have the potential to become the neural tissue and epidermis (Conklin [1]; Ortolani [3–5]; Reverberi [6]), the Golgi region characteristic of the larval epidermis was not mapped either in the a5.3 or the a5.4 blastomeres of the cleavage-arrested embryo. In the vegetal hemisphere of the cleavage-arrested embryo, the myofilament-dispersed region and the yolk region were very conspicuous (Figs. 11 and 12). The myofilament-dispersed region was clearly recognized in the B5.1 blastomeres in the cleavage-arrested embryo. It was mainly located at their periphery. The myofilaments, most of which form the bundles of myofilaments in the cleavage-arrested embryo, resembled the muscle elements observed in the normal larva, although the former were lacking in the Z-line and furthermore were not arranged in order. In the B5.1 blastomeres in the normal 16-cell embryo, no myofilament was recognized. Instead the large mitochondrial region was found in the B5.1 blastomeres of the normal 16-cell embryo as well as in the larval muscle cells, while it was not observed in the B5.1 blastomeres of the cleavage-arrested embryo (Figs. 11 and 12). In addition, the yolk mass observed in the large interior cytoplasm of the B5.1 blastomeres in the cleavage-arrested embryo closely resembled the endoderm in the normal larva. The B5.1 blastomere in the 16-cell embryo has the potential to form the endoderm, mesenchyme and muscle, however, the ultrastructural features in the mesenchme was not observed in the B5.1 blastomere of the cleavage-arrested embryo. On the other hand, the B5.2 blastomeres in the cleavage-arrested embryo did not produce any muscle elements although the B5.2 blastomeres have the potential to form the muscle as well as the B5.1 blastomeres (Figs. 11 and 12). In the B5.2 blastomeres of the normal 16-cell embryo, the mitochondrial region occupied most of their cytoplasm whereas this region was not found in the B5.2 blastomeres in the cleavage-arrested embryo. Instead, the blastomeres of the latter had quite a large region where the ER and the mitochondria were mingled at a similar density and so resembled the presumptive mesenchymal cells (Nishimura and Wake [7]), which are the one of the descendants of the B5.2 blastomeres. The yolk region was most conspicuous in the A5.1 and A5.2 blastomeres in the cleavage-arrested 16-cell embryo (Figs. 2a to 2d). The yolk region occupied almost the entire cytoplasm of these blastomeres and this ultrastructural feature applied to not only the A5.1 and A5.2 blastomeres in the normal 16-cell embryo but also to the endoderm in the normal larva. Although the A5.1 and A5.2 blastomeres in the 16-cell embryo have the potential to become the endoderm, notochord and neural tube, neither the vacuolation characteristic of the notochord nor the conspicuous ER region characteristic of the neural cells was found in the A5.1 and A5.2 blastomeres of the cleavage-arrested embryo.

IV. *Histochemistry of acetylcholinesterase*

Because acetylcholinesterase (AchE) is

Fig. 12. Comparison of distribution mode of organelles among cleavage-arrested 16-cell embryo (CB-16cell), normal 16-cell embryo (Nor-16cell) and normal larva.
Symbols are the same as in *Fig. 11*. The central section is the section with the nucleus and the lateral section is the section without the nucleus. Each blastomere and its developmental fate are discribed in the figure.
NER, nerve; EPI, epidermis; MUS, muscle; MES, mesenchyme; END, endoderm; NOT, notochord.

found histochemically only in the muscle cells of the tail of the developing ascidian larvae, AchE is thought to be a tissue-specific enzyme of the muscle cells (Whittaker [12]; Ohmori and Sasaki [17]; Durante [24]; Fromson and Whittaker [25]; Whittaker *et al.* [26]; Meedel and Whittaker [27]; Satoh [28]). We investigated the muscle differentiation based on the development of this enzyme in the cleav-



age-arrested 16-cell embryos of the ascidian, *Halocynthia roretzi*, by the sectioning method. It was confirmed that the AchE exists in the larval muscle cell and in close proximity to the plasma membrane of the muscle cell (Figs. 13c and 13d). The cleavage-arrested 16-cell embryo developed AchE in the B5.1 blastomeres. This enzyme was found not only in the interior cytoplasm but also in close proximity to the plasma membrane in the B5.1 blastomeres (Figs. 13a and 13b). But the B5.2 blastomeres in the cleavage-arrested embryo did not produce AchE. This finding well corresponded to the result of ultrastructural observation that the cleavage-arrested embryo formed many bundles of myofilaments in the B5.1 blastomeres but did not form any myofilaments in the B5.2 blastomeres.

Discussion

In the present study, we investigated the occurrence of the differentiation without the cleavage in the embryo of the ascidian, *Halocynthia roretzi*, from the ultrastructural point of view. As a result, it turned out that the differentiation without the cleavage occurred to a certain degree, because the extensive Golgi region characteristic of the larval epidermal cell surely appeared in the presumptive epidermal cells (b5.3 and b5.4 blastomeres) and bundles of myofilaments similar to the larval myofibrils were produced in the muscle lineage cells (B5.1 blastomeres) of the cleavage-arrested 16-cell embryo in spite of the irregular arrangements of the Golgi apparatus and myofilaments. These irregular arrangements may represent the developing forms of the cytoplasmic components, because the cytoplasmic components seem to be arranged irregularly at their first occurrence in development (Nishimura and Wake [7]; Terakado [29]). Otherwise, they may probably be due to the other drug effects than to the inhibition of cytokinesis by cytochalasin B. As to the other drug effects, the vacuoles between the blastomeres in the cleavage-arrested embryo are considered to be attributed to the inhibition of the intercellular contacts by cytochalasin B (Meller and El-Gammal [30]) and the interference of the tunica formation is probably due to the inhibition of secretion by cytochalasin B (Williams and Wolff [23]). It would be necessary to examine whether the irregular arrangements of the Golgi apparatus and of the myofilaments were evidently caused by the cytochalasin B. Crowther and Whittaker [31] reported that the extensive myofilaments which

Fig. 13. Acetylcholinesterase (AchE) activity in cleavage-arrested 16-cell embryo and normal larva.
All embryos and larvae were stained by the 'direct-colouring' thiocholine method (see Materials and Methods).
(a), (b): Cleavage-arrested 16-cell embryo (×200).
(a): (−) Toludine blue.
(b): (+) Toluidine blue.
Only the B5.1 blastomeres are stained with the reaction product. The AchE activity is found not only in the interior cytoplasm but also in close proximity to the plasma membrane.
(c), (d): Normal larva (×100).
(c): (−) Toluidine blue.
(d): (+) Toluidine blue.
Only the muscle cells are surely stained with the reaction product mainly in close proximity to the plasma membrane.
no, notochord; eq, epidermis; mu, muscle.

are less uniformly organized than in the normal hatched larvae are formed in the muscle lineage blastomeres of the cleavage-arrested 8-cell embryo (B4.1 blastomeres) and of the partial embryo resulting from the isolated B4.1 and B5.2 blastomere pairs. This time we obtained a similar result in the muscle lineage cells (B5.1 blastomeres) of the cleavage-arrested 16-cell embryo. However, we could confirm neither such muscle elements as observed in the normal larvae nor the AchE in the B5.2 blastomeres of the cleavage-arrested 16-cell embryo, though the B5.2 blastomeres are muscle lineage cells as well as the B5.1 blastomeres. The electrophysiological experiments in the cleavage-arrested embryo of the ascidian, *Halocynthia roretzi*, also revealed that, in the B5.1 blastomere of the 16-cell embryo, the muscle-type differentiation is apparently preferentially expressed, whereas in the B5.2 blastomere the differentiation into a non-excitable membrane apparently dominates and, furthermore, that for the B4.1 blastomere of the 8-cell embryo, the epidermal-type differentiation sometimes occurs despite this cell apparently not possessing the potential to become an epidermal cell (Hirano *et al.* [16]. But it would not be because of the difference of the ascidian species in use (Crowther and Whittaker [31] that the muscle-type differentiation is not expressed in the B5.2 blastomeres of the cleavage-arrested embryo of the ascidian, *Halocynthia roretzi*, for it was recently reported that the cell lineages do not seem to differ from species to species (Nishida and Satoh [32]. In any case, the processes of cell differentiation in the ascidian embryos are probably more complex than has been hitherto considered, as is suggested by the cell lineage analysis in the ascidian embryos by the intracellular injection of a tracer enzyme (Nishida and Satoh [32]). On the other hand, the regional cytoplasmic difference were indistinct in the cleavage-arrested 16-cell embryo, that is, its blastomeres, which were presumed to possess more than one possible developmental fate, did not develop the fine structure in a clear mosaic fashion. For example, the a5.3,4 blastomeres in the cleavage-arrested 16-cell embryo have the potential to become both the neural cell and epidermis (Conklin [1]; Ortolani [3–5]; Reverberi [6]). However, the differentiated ultrastructure in the a5.3,4 blastomeres was not a mosaic composed of structures characteristic of both the brain and epidermis, but the blastomeres resembled only the neural cells. Similarly, the A5.1,2 blastomeres have the potential to become the endoderm, notochord and neural tube, but these blastomeres closely resembled only the endodermal cells. The B5.1 blastomeres were analogous to the endodermal cells in the interior cytoplasm and to the muscle cells in the peripheral cytoplasm, despite having the potential to form the endoderm, mesenchyma and muscle. Though the B5.2 blastomeres have the potential to become the muscle and mesenchyme, they developed only a fine structure similar to the presumptive mesenchymal cells. And finally the b5.3,4 blastomeres which have only the potential to form the epidermis expressed the fine structures characteristic of the larval epidermis. In conclusion, so far as the differentiation of the fine structure is concerned, one or at the most two types of larval tissues appeared in each blastomere of the cleavage-arrested 16-cell embryo, even if they have more than two potential fates and the mosaic differentiation could not be well recognized. Our results concerning the "differentiation without cleavage" support the concept of localized cytoplas-

mic information which is responsible for the larval tissue development in the ascidian embryo, but it is suggested that the detailed mechanism of differentiation in the cleavage-arrested embryo must be further clarified because the cleavage-arrested blastomeres did not develop a mosaic ultrastructure.

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