Ultrastructural Development of Stalk-producing Cells in *Dictyostelium discoideum*, a Cellular Slime Mould

By R. P. GEORGE* AND H. R. HOHL

*Department of Microbiology and Pacific Biomedical Research Centre,
University of Hawaii, Honolulu, Hawaii 96822, U.S.A.*

AND K. B. RAPER

*Department of Bacteriology, University of Wisconsin,
Madison, Wisconsin 53706, U.S.A.*

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SUMMARY

Changes in the structure of the myxamoebae involved in the formation of the stalk of the fruiting body, or sorocarp, of *Dictyostelium discoideum* were investigated. Observed changes in the ultrastructure relating to cell differentiation include: (1) food vacuoles disappear and autophagic vacuoles take their place after cell aggregation; (2) an inclusion in the mitochondria disappears during the vegetative feeding stages; (3) the decrease in number of an electron-clear vacuole is coincident with the appearance of the 'prespore' vacuole in cells of the hind portion of the migrating pseudoplasmodium; (4) while the prespore cells diminish in size and their cytoplasm becomes electron dense during culmination, the myxamoebae destined to form cellular elements of the stalk swell and their cytoplasm finally disintegrates.

The slime coat that encircles the entire pseudoplasmodium remains after fixation and dehydration as a triple-layered membrane resembling cell membranes. At the start of culmination, the initial core of unwallied stalk-forming cells descends through the then vertically oriented cell mass. The polarization of cytoplasm in the horizontally oriented prestalk cells of the apical papilla is described, as well as the formation of the two-layered sheath around the stalk. The mass of so-called rearguard cells of the migrating pseudoplasmodium separates during culmination and a fraction of them form the basal disc while the remainder rise on the stalk just below the prespore mass.

INTRODUCTION

During the fruiting stage the myxamoebae of *Dictyostelium discoideum* construct, in the absence of any external energy source, a fruiting body, or sorocarp, composed of an upright, cellulose-ensheathed stalk bearing at its upper end a globose to lemon-shaped sorus containing the spores. The size of these fruiting bodies can vary within wide limits (Raper, 1941) and is controlled by several factors such as territory size and cell density (Bonner & Dodd, 1962) and critical mass (Hohl & Raper, 1964). The proportions of fruiting bodies, on the other hand, remain surprisingly constant over the entire size range (Raper, 1941; Bonner & Slifkin, 1949). The factors that control these proportions are unknown.

In this report we provide an analysis of the ultrastructural changes and the spatial distribution of cells and organelles during development. Particular emphasis has been placed on the prestalk cells which are responsible for the production of the cellulose tube, or sheath,

* Present address: Department of Zoology, University of Wyoming, Laramie, Wyoming 82070, U.S.A.

within which these cells vacuolate to form the stalk. The changes occurring in the prespore cells have been published previously (Hohl & Hamamoto, 1969*a*). This study was initiated on the basis of light-microscope findings described by Bonner (1944) and by Raper & Fennell (1952), and takes into account electron-microscope studies on *Dictyostelium* by Mühlethaler (1956), Gezelius & Rånby (1957), Mercer & Shaffer (1960), Gezelius (1961), Ashworth, Dundan & Rowe (1969), Maeda & Takeuchi (1969), Gregg & Badman (1970) and Kirk, McKeen & Smith (1971).

METHODS

Dictyostelium discoideum, strain NC-4(S2), was grown with the food organism *Escherichia coli* B/r on nutrient agar (1 % lactose, 1 % peptone, and 1.5 % Difco agar). After 43 h growth at 24° the myxamoebae were removed from the agar, separated from the bacteria by centrifugation, and placed on Millipore filters (Sussman & Lovgren, 1965) which were saturated with 1/60 M-Sorensen's phosphate buffer. The myxamoebae at the desired developmental stage were fixed on the filter by one of the following procedures:

(1) The filter was flooded with 1/60 M-Sorensen's phosphate buffer at pH 6.0 containing 1 % glutaraldehyde and 0.5 % paraformaldehyde for 1 h at room temperature, rinsed with buffer, and flooded with 1 % OsO₄ in the same buffer at pH 6.0 for 15 min (GF fixation);

(2) The specimens were fixed as above, but pretreated for 15 min in the Petri dish with vapour from a pad saturated with 2 % OsO₄ (O, GF fixation);

(3) The filter was flooded with an ice-cold mixture containing 6.25 % glutaraldehyde, 2 % osmium tetroxide, and 1 % phosphotungstic acid as described by Schäfer-Danneel (1967) and left for 1 h at room temperature (GOP fixation).

The pretreatment of myxamoebae with osmium vapour reduces the hydrophobic nature of the slime, allowing the specimens to be more easily immersed. Occasionally, however, there was some cell damage with this treatment. The fixed specimens were washed, dehydrated with ethanol followed by propylene oxide and embedded in Epon 812. Sections were poststained with Reynolds's lead citrate (1963) alone, or with saturated uranyl acetate (30 min at 60°) followed by lead citrate.

NaOH-insoluble stalk skeletons were prepared by autoclaving 5 min at 120° in 1 N-NaOH, neutralizing with HCl, washing and centrifuging. The skeletons were stained 15 min with 0.5 % BaMnO₄, then dehydrated and embedded in Epon 812.

RESULTS

During the entire life-cycle, the cytoplasm of the myxamoebae usually shows three distinct regions: (1) a peripheral region, just within the cell membrane, devoid of organelles, extending into pseudopodia; (2) a vacuolar region with occasional flat, smooth cisternae of the Golgi apparatus, concentration of vesicles and various types of vacuoles; (3) a non-vacuolar region containing most of the mitochondria and rough endoplasmic reticulum. Both vacuolar and non-vacuolar regions are in a perinuclear position. The three regions undergo qualitative as well as quantitative modifications during development.

Vegetative myxamoebae. The fine structure of the myxamoebae of *Dictyostelium discoideum* did not differ significantly from that of other cellular slime moulds. The nucleus, with its peripherally located, sometimes cross-shaped nucleolus (Fig. 1), was perforated by numerous pores 60 nm in diameter with a centrally located electron-dense granule. The mitochondria had tubular rather than lamellar cristae, and mitochondrial inclusions were rare at this stage (Fig. 2). The Golgi apparatus was composed primarily of numerous

Electron micrographs of the vegetative feeding stage.

Fig. 1. Vegetative myxamoeba feeding on *Escherichia coli* (B) with food vacuoles (F), contractile vacuole (C), and nucleolus (N). GOP fixation.

Fig. 2. Vacuolar cytoplasm (V) of vegetative myxamoeba from which run microtubules (small arrows). Electron-clear vacuole (E); mitochondrion (M). GOP fixation.

vesicles; typical stacks of smooth cisternae have been observed, but were rarely well developed (Fig. 2, 9). In addition, crystal-like bodies have been frequently observed which were located within the cisternae of the rough endoplasmic reticulum (Fig. 3). Lipoidal inclusions did occur in *D. discoideum* (Fig. 4) but were much less abundant than in *Acrasis rosea* (Hohl & Hamamoto, 1969*b*), *Acytostelium leptosomum* (Hohl, Hamamoto & Hemmes, 1968) or *Polysphondylium pallidum* (Hohl, Miura-Santo & Cotter, 1970).

Three types of vacuoles could be distinguished at this stage: (1) large, irregularly shaped vacuoles of homogeneous, low electron density (Fig. 1) that most likely corresponded to the contractile vacuole (water expulsion vesicles); (2) food vacuoles (Fig. 1) characterized by their content of bacteria at various stages of digestion (Hohl, 1965); (3) an electron transparent vacuole that was round or slightly ellipsoid, with a diameter ranging from 0.4 to 0.1 μm , and in which a thin coat of electron-dense material lined the inside of its limiting unit membrane (Fig. 2, 6) often obscuring the latter.

Microtubules were seldom observed in the non-vacuolar cytoplasm. They occurred in small groups within the vacuolar region of the cytoplasm (Fig. 2), and these groups have been observed converging upon an electron-dense centre.

Pseudopodia which were associated with cell movement and engulfment of food particles did not contain organelles. Their cytoplasm was granular at the periphery of the pseudopodia and fibrous in the central region, the fibrils being oriented parallel to the longitudinal axis of the pseudopodia.

The pseudoplasmodium. The aggregated myxamoebae in the pseudoplasmodium differed from those of the free-living vegetative stage in several particulars. (1) The number of food vacuoles decreased sharply as they were being expelled from the cells, whereas the number of autophagic vacuoles, characterized by their contents of cytoplasmic material such as mitochondria (Fig. 9), increased in number. Occasionally an autophagic vacuole appeared to engulf another vacuole along with surrounding cytoplasm (Fig. 7, 8). (2) The electron-transparent vacuole (Fig. 6), prominent in vegetative amoebae, remained in the prestalk cells of the fore-portion of the pseudoplasmodium, but was less frequently observed in the differentiating prespore cells of the rear portion. The fate of the disappearing transparent vacuoles has not been followed, yet there was a concomitant appearance of a new type of vacuole, the prespore vacuole (Fig. 4), in these prespore cells (Hohl & Hamamoto, 1969*a*). (3) Inclusions (Fig. 5) appeared in the mitochondria of myxamoebae after they ceased to feed and were found in all subsequent stages of development. In addition, dumb-bell-shaped mitochondria appeared. (4) From the beginning of aggregation to the later stages of sorocarp formation the differentiating cell mass was covered by the slime coat, but there appeared to be very little slime material between the cells in the aggregate. The one continuous

The cells in the developing sorocarp. (All electron micrographs unless noted.)

Fig. 3. Crystal within endoplasmic reticulum. O, GF fixation.

Fig. 4. Lipoidal inclusion (L) in a prespore cell on the outer surface of the sorogen. Prespore vacuole (P); slime coat membrane (T). O, GF fixation.

Fig. 5. Mitochondria with inclusion (arrow) in a prestalk cell. O, GF fixation.

Fig. 6. Electron-clear vacuoles in a differentiating stalk cell. Notice the fine periodic arrangement of the material on the inside of the membrane of these vacuoles. O, GF fixation.

Fig. 7. Autophagic vacuole engulfing another vacuole. Rearguard cells in the basal disc. O, GF fixation.

Fig. 8. Another example of phenomenon shown in Fig. 7.

Fig. 9. Autophagic vacuole (A) with mitochondrion (M) in differentiating stalk cell. Golgi apparatus (G). O, GF fixation.

element of the slime coat left after fixation and dehydration was a triple-layered membrane very closely resembling the cell membranes (Fig. 14). This membrane was clearly located outside the cells and we have not observed any connexions to the cell membranes. It could be traced intact around the entire sorogen surface (Fig. 15). There were granules, measuring 30 nm on the average, adhering to the slime-coat membrane and to the cell membranes of cells at the surface of the sorogen (Fig. 14). The granules and membranes were generally all that was seen on the upper portions of culminating sorogens, but on the lower portions of the supporting structure (e.g. basal disc) a fibrous material was present between the cells and the slime-coat membrane (Fig. 13).

Culmination. The proportions of the cells making up the rising sorogen are shown in a light micrograph (Fig. 10).

The top of the sorogen had the form of a rounded papilla covered by the slime coat, and structurally consisted of an outer layer of horizontally oriented and strongly polarized cells that formed a cylinder bounded on its inner surface by the cellulose stalk sheath which enclosed a core of isodiametric and incompletely differentiated stalk cells (Fig. 11). The sheath with its entrapped stalk cells extended upward into the papilla and downward through the prespore cells to the basal disc. Below the prespore cells are a group of cells, the rear-guard cells, which at the stage illustrated closely resembled the prestalk cells in staining and ultrastructure. We will discuss the different parts in the following sequence: polarized pre-stalk cells and their relationship to sheath formation and the slime coat; the differentiation of the stalk cells and deposition of the inner layer of the sheath; and the differentiation of the basal disc.

The polarized cells of the papilla extended radially outward and slightly upward from the cellulose stalk sheath to the periphery of the cell mass (Fig. 11). Their cytoplasmic contents, starting at the stalk sheath and moving outward, were arranged as follows. Just behind the plasma membrane bordering the sheath there was a zone 0.5 to 1.0 μm wide, devoid of cell organelles but filled with a finely fibrillar material (Fig. 16). Sections show that at least some of these fibrils ran at right angles to the cellulose fibrils and approximately parallel to the cell surface (Fig. 18). Next to this pseudopodial-like region there was a zone where mitochondria dominated, and this zone bordered the vacuolar cytoplasm which contained prominent autophagic vacuoles. The nucleus was outward from this, and the remainder of the cytoplasm was less clearly stratified. The fine cytoplasmic fibrils could also be seen at the cell ends nearest the slime coat (Fig. 14). The autophagic vacuoles often occurred at the same distance from the sheath. Thus in longitudinal section through the rising sorogen (Fig. 11) they were lined up parallel to the sheath.

Strands of cellulose, which constituted the structural component of the stalk sheath, could be observed adjacent to, but outside, the plasma membrane of the polarized cells (Fig. 16).

Fig. 10. Light micrograph of longitudinal section of sorogen stained with Toluidine blue. Basal disc (B); dark-staining prespore cells (P); rearguard cells (R); stalk (S); prestalk cells (U). GF fixation.

Fig. 11. Light micrograph of the papilla at higher magnification from Fig. 10. Notice the alignment of vacuoles (arrows) parallel to and a distance away from the sheath (P). Oriented, elongated and polarized prestalk cells (O); stalk (S); prestalk cells not yet elongated (U). GF fixation.

Fig. 12. Longitudinal section through the upper portion of the stalk. Autophagic vacuoles (A) have enlarged greatly and occupy the main portion of the stalk cells. The black deposits represent acid phosphatase activity. Sheath (P); rearguard cells (R).

Fig. 13. Wall-like material (W) underlies the slime-coat membrane (T), and is apparently secreted by these rearguard cells of the basal disc. Granule (g). O, GF fixation.

Fig. 14. The triple-layered slime-coat membrane (T) at the level of the papilla. Granule (g); fibrils in cytoplasm (f). O, GF fixation.

Fig. 15. Left side and bottom of the basal disc of a sorogen in mid-culmination. Section plane parallel to long axis of stalk. Thick outer layer of cellulose sheath (p) becomes thin and disappears as one follows it into the basal disc. Slime-coat membrane (T) covers entire sorogen, as well as under the basal disc (BT). Enlarged vacuole of stalk cell (S). O, GF fixation.

Part of the membrane appeared to be tightly attached to the sheath and a cytoplasmic strand remained extended between the sheath and the cells when they were accidentally parted (Fig. 17). The cellulose microfibrils observed in the outer sheath layer next to the prespore cells were oriented longitudinally, i.e. parallel to the stalk axis and to the movement of the prestalk cells (Fig. 16), whereas the fibrils of the inner sheath layer, as will be discussed later, were randomly oriented (Fig. 19).

The sheath thinned gradually toward the tip of the papilla, then disappeared. The apex of the papilla was examined for the presence of structurally unusual cells which might be 'organizers' but none was found. The prestalk cells in the top of the papilla were roundish, and enlarged autophagic vacuoles had become more conspicuous. This trend continued as one observed the stalk cells farther down in the stalk, where eventually the autophagic vacuoles occupied the major portion of the cells. Concomitantly, the cytoplasmic organization gradually diminished (Fig. 10–12, 15). In addition, the cells in the stalk became swollen, and their cytoplasm was much less dense than in prestalk cells. The swollen cells assumed a polyhedral shape, most likely caused by the close packing, and eventually each cell was surrounded by a single-layered wall (Fig. 20). At some time before beginning to lay down walls, the differentiating stalk cells deposited a cellulose layer on the inner surface of the sheath of parallel cellulose microfibrils. This inner layer of the sheath differed from the outer one in: (1) the fact that it did not reach as high in the papilla as the outer layer, (2) the texture of the microfibrils, which were randomly oriented rather than parallel, (3) the fact that it was deposited on to the external layer, i.e. after the external layer had been formed.

In the earliest stages of culmination examined, the papilla contained a peripheral zone of elongated prestalk cells and a core of rounded prestalk cells which had not yet formed walls. The core cells were easily recognized by their large vacuoles and low electron density. The core cells eventually came to rest upon a group of rearguard cells covering the substratum. The rearguard cells surrounding the lower portion of the stalk core then began to vacuolate and surround themselves with walls, thus forming a basal disc. The cellulose sheath enclosing the stalk only reached down to the upper limits of the basal disc and then became thinner and gradually disappeared (Fig. 15).

While some of the so-called rearguard cells remained adjacent to the substratum and differentiated, others adhered to the prespore cell mass in the rising sorogen (Fig. 10). These rearguard cells actually contacted the prestalk cells by means of a layer of cells, variable in thickness, which separated the prespore cells and the stalk sheath.

The prespore and the prestalk cell regions intermingled only slightly at their junctions and remained segregated without any observable physical barriers. There was no clear evidence of specialized sites of attachment between cells in the pseudoplasmodium.

DISCUSSION

The mitochondrial inclusion in *Dictyostelium discoideum* is observed in myxamoebae during spore germination (Cotter, Miura-Santo & Hohl, 1969), aggregation, migration and culmination (i.e. non-feeding stages). It is only rarely seen in vegetative myxamoebae. Similar inclusions have been observed in *Acanthamoeba castellanii* during encystment, or when it was starved (Bowers & Korn, 1969). The absence or presence of the inclusion might possibly reflect changes in the state of the mitochondrial DNA.

The electron-clear vacuole represents another candidate for precursor of the prespore vacuole (cf. Hohl & Hamamoto, 1969*a*). It exists in all cell types, including mature spores

(Cotter *et al.* 1969), but decreases in number in prespore cells of the sorogen. However, the origin of the prespore vacuoles needs further clarification.

A scanning electron microscope study of freeze-dried specimens of *Dictyostelium discoideum* (George *et al.* 1970) showed that the actual slime coat is more complex than the simple layered membrane remaining after fixation and dehydration (Fig. 14). It is significant, however, that the slime-tube network enclosing the cells of another aggregating organism, *Labyrinthula*, when fixed and dehydrated, are triple-layered membranes (Porter, 1969; Stey, 1969) like the slime coat of *D. discoideum*. Aschner & Kogan (1959) cite evidence suggesting that the slime pathway or tube of *Labyrinthula* might itself carry signals directing cells to food elsewhere in the network. In *D. discoideum* it is possible that the prestalk cells at the surface of the papilla elongate inward and have their cytoplasm polarized in response to a signal from the slime coat. Although there is no direct evidence of slime-coat involvement in organizing the distribution of cells or even the induction of differentiation in a pseudoplasmodium or sorogen, the possibility deserves further investigation, since it accommodates the radially symmetrical development of the sorogen and this air-sorogen interface provides a clear-cut reference surface for development.

No storage products for sheath and wall synthesis were discovered within the cells during this study. The polarization of the elongated cells, however, conveniently places the most likely producer of polysaccharide building blocks (the autophagic vacuoles) and the energy producers (mitochondria) very near the site of stalk-sheath synthesis.

The following evidence indicates that the site of synthesis of sheath and wall cellulose is located on, or very near to, the cell membrane. (1) The strong attachment of sheath and elongate cells (Fig. 17) suggests a physical connexion, perhaps even synthetic sites, on the cell membrane. (2) We have not found ultrastructural evidence that any material passes directly through the cell membrane to the stalk sheath or cell walls. (3) Secretory vesicles are not found near the site of sheath and wall synthesis. (4) The clear separation of the cellulose sheath from the stalk cell walls, and the walls from each other following treatment with NaOH (Fig. 20) indicates that cellulose-synthesizing sites are bound to the cell surface rather than free in the sheath and wall matrix, since in the latter case one would expect an intermingling of cellulose fibres from sheath and walls. (5) A radioautographic study indicates that initial incorporation of glucose into the polymer occurs in or very close to the sheath and cell walls and not deep within the cells (George, 1969).

The outer layer of the cellulose sheath is made up of parallel microfibrils (Gezelius & Rånby, 1957; George & Hohl, 1969) which orient along the main axis of the stalk (Mülethaler, 1956). The upward movement of the elongate cells perhaps provides the directional stress necessary for parallel orientation of the cellulose fibrils in the sheath. Indeed,

Fig. 16. The elongated prestalk cells (O) dragging upward (toward right upper corner) over the parallel cellulose fibrils of the outer stalk sheath layer (p) in the papilla. Notice the cell ends pulled out of shape on the downward side. Fibrous cytoplasm (f); stalk cell (S); mitochondria (M). O, GF fixation.

Fig. 17. The elongated prestalk cells (O) accidentally pulled away from the sheath (p), leaving cell extensions (arrows) attached on the downward ends. GF fixation.

Fig. 18. Cross-section of the stalk in the papilla, showing cytoplasmic fibrils (f) in the prestalk cells running at right angles to the parallel cellulose fibrils of the sheath (p) which run perpendicular to the plane of this micrograph. Stalk cell (S). O, GF fixation.

Fig. 19. Longitudinal section of the stalk with outer parallel cellulose sheath layer (p) and inner random cellulose layer (i). GF fixation.

Fig. 20. NaOH-extracted stalk. Outer sheath layer is missing. Inner sheath layer (i); stalk cell walls (W).

longitudinal sections of the papilla give an impression of considerable tension and friction between the cells and the outer surface of the sheath (Fig. 16, 17).

The inner layer of the sheath is probably produced by stalk cells. Since the stalk cells do not move upward or downward after the stalk core has reached the substratum, the cellulose texture is random like that of the cell walls (Fig. 19).

A comparison of the previous ultrastructure study on culmination in *Acytostelium leptosomum* (Hohl *et al.* 1968) with the study presented here helps to define some characteristics of stalk formation. The structures in both generally are somewhat similar, except that during culmination *A. leptosomum* shows no separate prestalk and prespore cell masses, the same myxamoebae functioning first as horizontally elongate (from slime coat to lengthening stalk) prestalk cells and subsequently differentiating as globose spores. In both systems the stalk has a lumen, but in *A. leptosomum* it is small and contains no cells, whereas that in *Dictyostelium discoideum* becomes filled with compacted and strongly vacuolate cells. The lifting force during culmination of *D. discoideum* is probably supplied in part by the elongated prestalk cells, like the force in *A. leptosomum*, although the mechanism remains unclear, and in part by the pressure developed by the swelling stalk cells (Raper & Fennell, 1952).

The basal disc is a secondary development of the stalk peculiar to *Dictyostelium discoideum*. It results when the initial core of stalk cells becomes embedded in a mass of cells, resembling prestalk cells, which remain spread on the substratum after the major portion of the sorogen has risen into the air. The innermost layer of these cells differentiate into stalk-like cells first, possibly under the influence of the adjacent stalk cell core, and then the differentiation spreads progressively outward until all the cells have become strongly vacuolate and walled in.

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