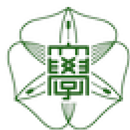

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Scanning electron microscopic studies on cell surfaces of *Dictyostelium discoideum*: osmotic and pH effects during fixation

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Fixation conditions can have a significant effect on fine structure. Osmotic and pH effects which produce artifacts on cells during fixation make adequate interpretation of findings impossible.

The authors have studied the effect of fixation at different osmotic pressures and different pH's on *Dictyostelium discoideum* myxamoebae with scanning electron microscopy. Glutaraldehyde solutions with various concentrations of vehicle and different pH's were used.

Optimum preservation of the micro structure with glutaraldehyde fixative was achieved when the osmolality of the vehicle was approximately 110 milliosmols and pH was 6.3-7.3. On the cells fixed at pH 5.8, numerous small vesicles were observed.

Aggregation in the cellular slime mold *Dictyostelium discoideum* has been shown to involve biochemical changes at the cell surface (GERISCH, 1968; WEEKS and WEEKS, 1975; MALKINSON and ASHWORTH, 1973; MALKINSON *et al.*, 1972), but the question of whether an aggregation also involves morphological alterations at the cell surface remains unanswered. Light microscopic studies have revealed many characteristics of cell forms (KOBILINSKY *et al.*, 1976; GARROD and BORN, 1971), but little is known as to the fine structure of cell surfaces of the amoebae during aggregation (MOLDAY *et al.*; 1976; GEORGE *et al.*, 1970; ROSSOMANDO *et al.*, 1974). Fixation procedures, as employed for scanning electron microscopy, often reform or alter the appearance of the cells (CLARK and GLAGOV, 1976).

In this paper we describe the effects of various osmotic and pH conditions on the surface morphology of myxamoebae, as detected by scanning electron microscopy.

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Materials and Methods

Organism and culture conditions

D. discoideum cells NC-4 (haploid) were grown in association with *E. coli.* The late log-phase cells were harvested in buffered Bonner's salt solution freshly made up and washed four times with the same buffer (OCHIAI and HASE, 1976). The cell suspensions of 0.6 m*l* at a final concentration of 2.5×10^7 cells per m*l* were put into glass dishes of 60 mm having several glass cover slips and allowed to settle for about 60 or 90 min at 22 C.

Fixation

The myxamoebae on 10-mm cover slips in Petri dishes were exposed gradually to 2 per cent glutaraldehyde in phosphate or cacodylate buffers having osmotic pressures of various strengths or having various pH's. The volume of fixative solutions was 4 m*l*.

The different kinds of glutaraldehyde-based fixatives utilized were: (1) 2% glutaraldehyde (GA) in 0.2 M phosphate buffer (PB) (pH 7.3). (2) 2% GA in 0.15 M PB (pH 7.3). (3) 2% GA in 0.1 M PB (pH 7.3). (4) 2% GA in 0.05 M PB (pH 7.3). (5) 2% GA in 0.15 M cacodylate-HCl buffer (CB) (pH 7.3). (6) 2% GA in 0.13 M CB (pH 7.3). (7) 2% GA in 0.1 M CB (pH 7.3). (8) 2% GA in 0.08 M CB (pH 7.3). (9) 2% GA in 0.05 M CB (pH 7.3). (10) 2% GA in 0.15 M PB (pH 7.3). (11) 2% GA in 0.15 M PB (pH 6.8). (12) 2% GA in 0.15 M PB (pH 6.3). (13) 2% GA in 0.15 M PB (pH 5.8).

Dehydration and preparation for scanning

After the fixation in the above fixatives, the cells were washed several times with 0.15 M phosphate buffer (pH 7.3) for about 2 hr at 19–22 C, post-fixed in 1 per cent OsO_4 in 0.15 M phosphate buffer (pH 7.3) for 60 min at 4 C, and washed with distilled water. The cells were dehydrated in a graded series (50%, 60%, 70%, 80%, 90%, 100%, 100%) of ethanol and then in a series of isoamyl acetate in ethanol up to 100% isoamyl actate. Thereafter they were transfered into liquid CO_2 in a critical-point drying apparatus (Hitachi HCP-1) and, after two rinses in CO_2, the temperature of the closed system was raised to 42 C according to the critical-point method (ANDERSON, 1950). The resulting dry specimens were lightly coated by an ion coater IB-3 (Eico Engineering Co.) and stored in a dessicater ready for microscopy. A Hitachi-Akashi (MSM-4) scanning electron microscope, operated at 10 kV, was used.

Results

No specializations other than large pseudopodial structures were observed by light microscopy. However when cells were examined by scanning electron microscopy after mild fixation, small slender projections on the cell surface were observed.

Comparison of different osmotic pressures during fixation.

The cells in Plate I a and I b show many slender projections (filopodia) attached to the substrate around their bases and attached to the surfaces of the other cells. These filopodia vary both in length and in number per cell. The residual upper cell surfaces waved except for the filopodia. The fixation in the very hypertonic buffer (0.2 M phosphate buffer) detached a considerable number of cells from the substrate. The cells in suboptimal fixatives (vehicle osmolality; 60–210 milliosmols) had extensive areas of comparatively smooth surfaces (Pl. I e and I f), showed leading lemella with blunt raffling membranes (Pl. I f) and rarely bleb (PORTER *et al.*, 1973)-like extrusions (Pl. I c). The cells in Fig. 1 c show a large bubble with a secondary-formed cave. The likely cause of such a bubble is osmotic swelling.

As noted many times (RUBIN and EVERHART, 1973), animal tissue cells round up during mitosis and attach to the substrate by a complex system of filopodia. The cells of the cellular slime mold also rounded up with stretched filopodia in the late prophase (Pl. II a) and at the metaphase the exposed surface of the cell showed peculiar folds (Pl. II b). These folds may accomodate large areas of surface membranes of cells which have developed many filopodia.

Comparison of the different pH's during fixation.

A series of cells from the same culture in Petri dishes at 22 C were fixed in 2 per cent glutaraldehyde buffered at various pH's (pH 5.8, 6.3, 6.8, and 7.3). The concentration of the phosphate buffers was the same (0.15 M) at every pH. The finding in such experiments are shown in Plate III. The fixative solution at pH 5.8 caused numerous "Bubblings" from the whole surface of practically every cell (Pl. III d). In the present experiments the question of whether this phenomenon is due to the effect of glutaraldehyde in low pH buffer or to only low pH was unanswered. Three fixatives adjusted to pH 7.3, 6.8, and 6.3 (2% GA in 0.15 M phosphate buffer) gave indistinguishable results (Pl. III a–III c). Morphological effects due to pH during fixation must be studied further.

Discussion

One of the most difficult problems in scanning electron microscopy is to evaluate the quality of fixation; to decide how closely the fixed images approximate those of the living cell. BOYDE and VESELY (1972) employed phase-contrast microscopy followed by SEM examination of the same cells to compare the SEM images carefully with those observed under light microscopy. Our comparison made by phase-contrast microscopy before and after fixation revealed some alteration of cell form; *e.g.* when actively-migrating myxamoebae were exposed to a perfusion of fixatives the cells appeared to result in the go-back movement of leading filopodia on cells.

The osmolality of aldehyde-based fixatives is of considerable importance for preservation of the surface ultrastructure (BRUNK *et al.*, 1975; BOYDE and VESELY, 1972). Recent evidence (BOYDE and VESELY, 1972) shows that the total osmolality of the fixatives is of less importance than the effective osmotic pressure contributed by the fixative vehicle. Optimum fixation under present conditions in terms of the SEM image occurs with a fixative (2% GA in 0.05 M phosphate buffer) which has vehicle osmolality approximately equal to the osmolality of the cells. As BOYDE and VESELY (1972) and BRUNK *et al.* (1975) have pointed out, and we confirmed, the osmotic pressure of the buffer in a fixative should be nearly equal to the osmotic pressure of the cells. Although COHEN and SHAYKH (1973) indicated that the dilute osmium solution is far superior for the preservation of external morphology of myxamoebae, our experience was totally unsatisfactory because of the considerable detachment of cells from substrates by rounding up of cells due to the hypotonic fixation.

The form of myxamoebae grown under our control conditions has proven reproducible over the course of ten independent experiments. Yet these cells assume very different forms in response to specific changes in the culture environment (unpublished results). Thus, it is clear that culture conditions must be standardized within each experiment in order to compare SEM observation of cell form. In addition, it is important to know the changing character of the cell through the course of the life cycle of the cellular slime mold.

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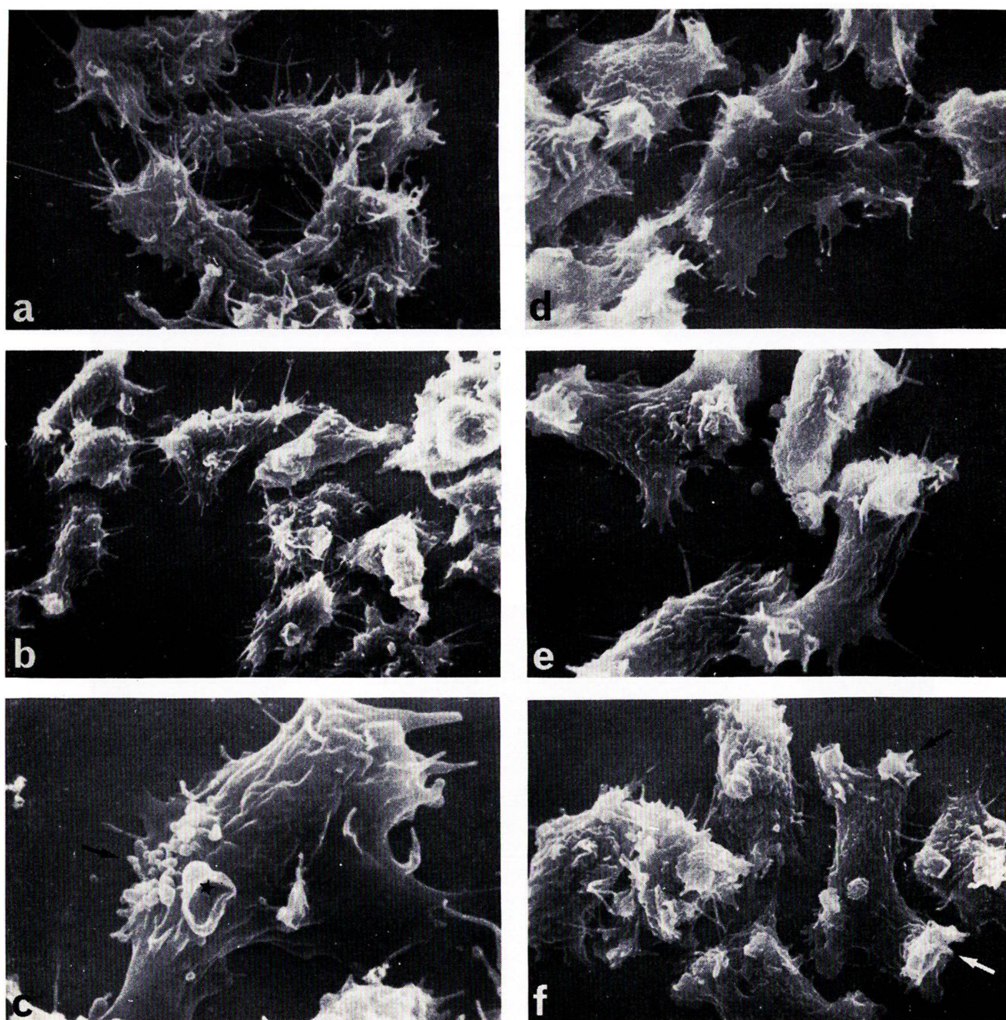
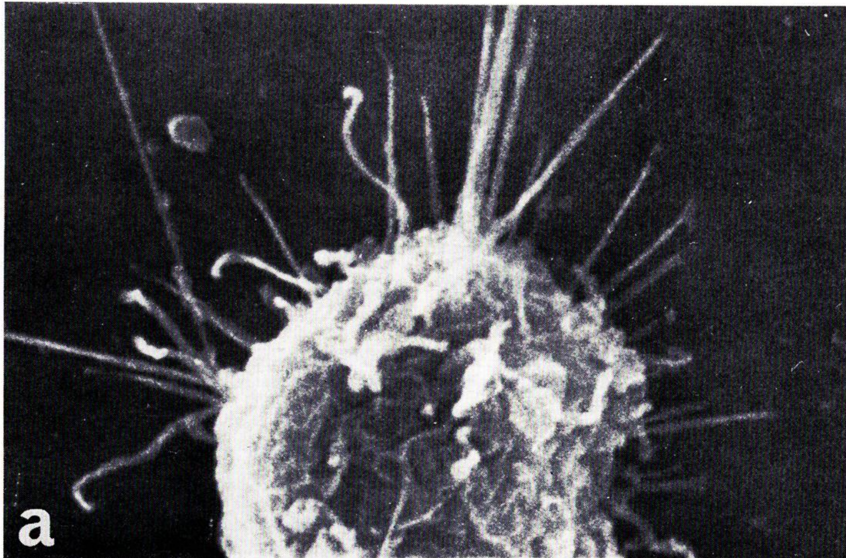


Plate I. Myxamoebae fixed in fixatives having various osmolalities. Cells were harvested, allowed to settle on glass in suspension, and fixed in 2% glutaraldehyde (GA) in phosphate or cacodylate buffers having various osmotic pressures. (a) 2% GA in 0.15 M phosphate buffer pH 7.3. × 4148. (b) 2% GA in 0.13 M cacodylate buffer pH 7.3. × 1160. (c) 2% GA in 0.10 M phosphate buffer pH 7.3. × 3368. Note bleb-like extrusions (arrow) and a large bubble (*) with a secondary-formed cave. (d) 2% GA in 0.10 M cacodylate buffer pH 7.3. × 1933. (e) 2% GA in 0.08 M cacodylate buffer pH 7.3. × 1933. (f) 2% GA in 0.05 M cacodylate buffer pH 7.3. × 2406. Note lamellipodia (arrows) at the leading edges of the cells.



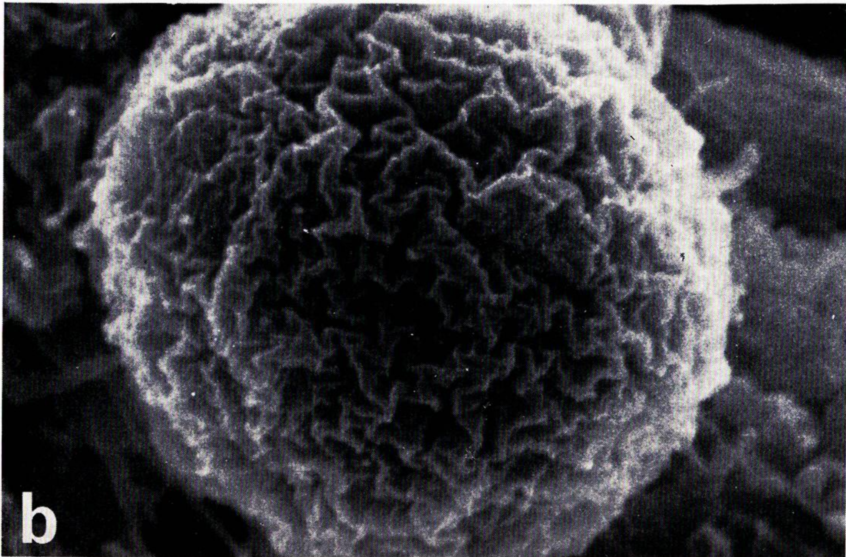


Plate II. Myxamoebae in stages of late G_2 (a) and mitosis (b). Specimens were prepared as described in legends to Plate I. (a) the cells fixed in 2% GA in 0.2 M phosphate buffer. They are attached to the substrate by many filopodia. × 17850. (b) the cells, fixed in 2% GA in 0.15 M phosphate buffer, are probably in a stage of mitosis. The cell surface has been totally surrounded with peculiar folds. × 14933.

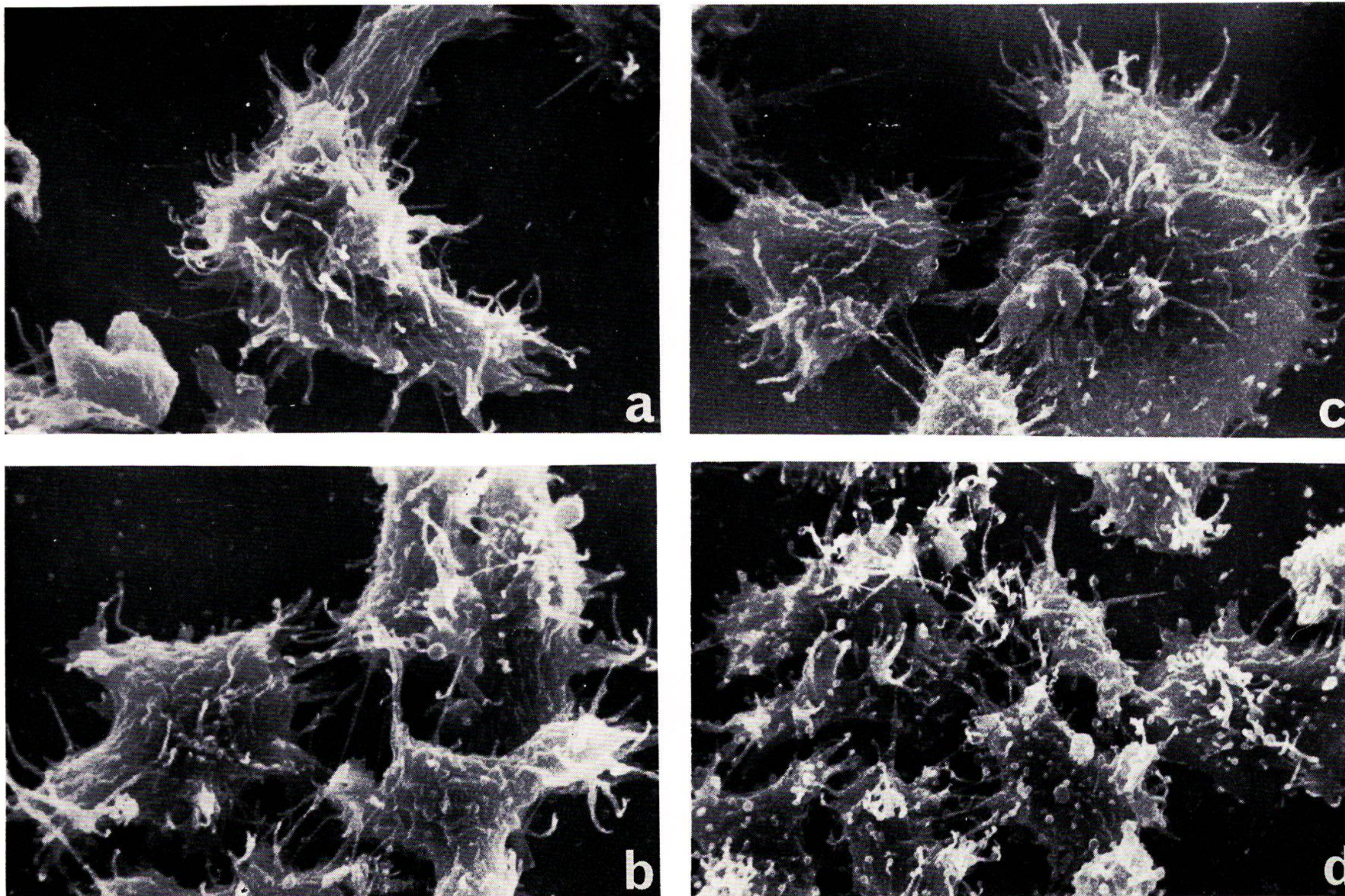


Plate III. Myxamoebae fixed in 2% glutaraldehyde buffered respectively at pH 5.8, 6.3, 6.8, and 7.3 with 0.15 M phosphate buffer. Specimens were prepared as described in legends to Plate I. (a) 2% GA buffered at pH 7.3. × 2697. (b) 2% GA buffered at pH 6.8. × 2841. (c) 2% GA buffered at pH 6.3. × 2697. (d) 2% GA buffered at pH 5.8. × 2589. Note numerous small vesicles.