STRUCTURAL ORGANIZATION ASSOCIATED WITH PSEUDOPOD EXTENSION AND CONTRACTION DURING CELL LOCOMOTION IN DIFFLUGIA

A. WOHLMAN* and R. D. ALLEN†
Department of Biology, Princeton University, Princeton, New Jersey, U.S.A.

SUMMARY

In Diffugia corona, a free-living amoeboid cell, locomotion is hampered by a heavy shell or test made of sand grains and other debris. Locomotion involves pseudopod extension, attachment to the substratum, and forcible pseudopod retraction which pulls the shelled cell body forward.

When observed through a polarizing microscope, the extending pseudopodia appear isotropic or very weakly birefringent. Upon attachment to the substratum a positively birefringent fibrillar array develops rapidly at the attachment point and extends from this region back to the cell body within the test. These birefringent fibrils extend through and parallel to the long axis of the pseudopod. As the pseudopod retracts, the birefringent fibrillar array disappears, and hyaline blebs, suggestive of syneresis, appear on the pseudopodial surface. The birefringent fibrils correspond in position and approximate diameter (1 μ) to refractile fibrils visible with the Nomarski differential interference microscope.

Individual organisms were fixed for electron microscopy at a time when the pseudopodia were firmly attached to the substratum. Electron-microscopic examination of thin sections of pseudopodia revealed many 1-μ bundles of intimately associated, aligned, 55-75 Å microfilaments. The orientation and size of the bundles indicate that they probably correspond to the birefringent, refractile fibrils observed in living cells. Microfilaments have also been observed both as randomly oriented and dispersed cytoplasmic components, and as aligned filaments in the ectoplasm adjacent to the plasmalemma.

During pseudopod extension with sporadic streaming, birefringent 'flashes' have been observed at the front of the pseudopod. These flashes are believed to represent a photo-elastic phenomenon.

INTRODUCTION

Attempts to analyse the mechanisms underlying amoeboid movement and cytoplasmic streaming have, for many years, been focused on the search for fibrils or filaments which, upon contraction, could provide the required motive force. The search has been largely limited to the large, free-living, naked, carnivorous amoebae (see Allen, 1961a). These ideas can be traced back to the early nineteenth century. Following Dujardin's (1835) description of the cytoplasm of amoeboid cells ('sarcodex')

* Present address: Bell-Craig Laboratories for Medical Research, 451 Alliance Avenue, Toronto 9, Ontario, Canada.
† Present address: Department of Biological Sciences, State University of New York at Albany, Albany, New York, U.S.A.
as contractile, and Brücke's (1861, 1862) proposal that the cytoplasm consisted of a contractile framework, Engelmann (1869, 1875, 1879) further postulated the existence of 'Inotagmen', uniaxially birefringent, contractile elements capable of undergoing form changes to produce the force responsible for cytoplasmic movements.

The concept that contractile fibrils or filaments might provide the motive force for cell movements and cytoplasmic streaming has also been discussed considerably in the current literature (for example, see Seifritz, 1929; Meyer, 1929; Monné, 1948; Loewy, 1949; Goldacre & Lorch, 1950; Frey-Wyssling, 1953; and Allen, 1961b).

The search for fibrils at the light-microscopic level in living cells has not been very fruitful; on the other hand, the electron microscope has revealed a number of fibrillar elements, ranging between 30 and 80 Å in diameter, present in fixed cells (Randall & Jackson, 1958; Sotelo & Trujillo-Cenóz, 1959; De Petris, Karlsbad & Pernis, 1962; Yaqui & Shigenaka, 1963; Danneel, 1964; Nachmias, 1964, 1966; Wohlfarth-Bottermann, 1964a, b; Wolpert, Thompson & O'Neill, 1964; Baker, 1965; Komnick & Wohlfarth-Bottermann, 1965; McManus & Roth, 1965; Nagai & Rebhun, 1966; Cloney, 1966; Drum & Hopkins, 1966; Masurovsky, Benitez & Murray, 1966; Overton, 1966; Taylor, 1966).

The present paper demonstrates the existence of fibrils in pseudopodia of living testaceans and shows that these fibrils form during pseudopod extension, apparently to participate in forcible pseudopod retraction during cell locomotion. Closer examination with the light and electron microscopes has enabled us to characterize the fibrils as microfilaments.

**MATERIALS AND METHODS**

The organism used for this study was *Diffugia corona* (Wallich), a fresh-water testacean. The order Testacea, part of the class Sarcodina, contains those amoebae which inhabit a single-chambered shell. For general information concerning the testaceans the reader is referred to the works of Leidy (1879), Mast (1931), Deflandre (1953) and Jepps (1956).

Most of the organisms were obtained from the surface mud or ooze at the bottom of sphagnum bogs in the pine barrens of south-eastern New Jersey. *Diffugia corona* was cultured in either the natural waters in which it was found or in Marshall's medium (0.05 mM MgSO$_4$·7H$_2$O; 0.5 mM CaCl$_2$; 0.15 mM K$_2$HPO$_4$; 0.11 mM KH$_2$PO$_4$ in distilled water), supplemented with diatoms and finely-ground quartz sand. The cultures were maintained at 18–20 °C under a light–dark cycle consisting of 12 h of light and 12 h of dark.

Light microscopy involved the use of two contrast-generating systems: polarized light and Nomarski differential interference optics (Nomarski, 1955). The optical components were manufactured by Carl Zeiss (Oberkochen) and were mounted in a Photomicroscope I. Photomicrography on 35-mm film was accomplished by means of the internal camera and accompanying automatic exposure device of the Photomicroscope. Because of the limited light available during observation of the organism between crossed polars, photographic records were made on Kodak Plus-x film processed with Diafine developer which increased the ASA rating 6–9 times.
Pseudopods of Diffugia

(Baumann Photochemical Corp., Chicago, Illinois). The more favourable light intensity afforded by ordinary bright-field and differential interference microscopy permitted the use of a finer-grained film, Adox KB-14, which was also developed in Diafine.

Motion pictures were taken with an Arriflex 16-mm camera equipped with either a synchronous motor drive for 8 frames/sec or variable-speed d.c. motor. When framing rates of 2/sec or less were desired, the Arriflex DOM time-lapse apparatus was used, Kodak Plus-x negative film, developed in Diafine, was used for motion pictures of the organism in polarized light. In the case of bright-field and differential interference microscopy, the finer-grained British Kodak High Contrast Pan film gave the most satisfactory results. Analysis of 16-mm film was accomplished with a modified Kodak 'Analyst' projector ('Photo-optical data analyzer', L-W Photo, Inc., Van Nuys, California).

For electron microscopy, individual organisms were placed in a small drop of Marshall's medium and allowed to attach to the substratum. Fixation was achieved by flooding with 3·8% glutaraldehyde in 0·13 M phosphate buffer at pH 7·2. After 20 min at room temperature, the cell was washed 3 times in Marshall's medium, post-fixed in 1% osmium tetroxide (1 h), washed again and dehydrated through an ethanol series. After the specimen was cleared in propylene oxide it was allowed to equilibrate overnight, at room temperature, in a 1:1 mixture of complete Epon resin (including hardener) and propylene oxide. Following equilibration, the cell was embedded in Epon (Luft, 1961) and sectioned with a Huxley ultramicrotome. Sections stained with uranyl acetate (Stempak & Ward, 1964) and lead citrate (Reynolds, 1963) were examined in the Hitachi electron microscopes HS-7 and HU-11.

RESULTS

Pattern of pseudopodial movement

*Diffugia corona* moves by extending cylindrical pseudopodia which attach to the substratum, then forcibly retract, pulling the shelled cell body forward. In a typical sequence in the movement of *D. corona*, pseudopodia can be seen to extend above the substratum, swing to one side or the other, and subsequently settle down and attach. Attachment of the pseudopod to the substratum is quite firm, as evidenced by the fact that small, membrane-bound fragments are often left on the substratum following pseudopod detachment. These membrane-bound fragments show independent movement for indefinite periods despite their lack of a nucleus. Numerous pseudopodia may be put forth and the force determining the velocity of locomotion is apparently the vector sum of the forces exerted by the contracting pseudopodia. Competition between pseudopodia extended at approximately 180° with respect to each other results in detachment and retraction of the less firmly attached pseudopod, while contraction of the dominant pseudopod determines the direction of the organism. Two adjacent pseudopodia from the same organism will often fuse if they touch at any point along the pseudopodial surface. A cytoplasmic bridge is frequently seen
to form at the proximal end of two adjacent pseudopodia. Membrane fusion has never been observed upon contact of pseudopodia from different organisms.

**Pseudopod extension and cytoplasmic streaming**

Cytoplasmic streaming as observed in *D. corona* closely resembles that described for *Amoeba proteus* and *Chaos carolinensis*. Endoplasm streams but the hyaline cap frequently seen at the front tip of naked amoebae is often not evident in *Difflugia*. In many instances cytoplasm has been observed to stream into an extending pseudopod and subsequently to continue streaming distally as the pseudopod itself retracts and is withdrawn into the cell body. Consequently, bulbous formations sometimes appear at the pseudopodial tip. In Fig. 1 the arrow identifies and follows a single particle flowing in the endoplasmic stream while the pseudopod itself is retracting. Reversal of the direction of streaming initiates at the proximal end of the pseudopod (as in *C. carolinensis* and *A. proteus*) and the wave of reversal propagates distally. Cytoplasm, streaming out of a retracting pseudopod, can flow in a U-shaped pattern and enter another pseudopod without circulating through the cell body. The velocity of streaming has been observed to range from 2 to at least 15 μ/sec and is highly variable.

Very rapid, brief flashes of relatively strong positive birefringence have been noted to occur in normally weakly birefringent extending pseudopodia undergoing sporadic cytoplasmic streaming. Such flashes are more easily seen in projected films than in single frames printed from the film.

**Fibril formation upon pseudopod attachment**

When observed between crossed polaroids, the pseudopods appear very weakly positively birefringent as they extend from the cell body (Fig. 2A). Upon attachment to the substratum, at any point along the pseudopod, strong positive birefringence develops rapidly at the attachment area and spreads both toward the test and toward the front tip of the pseudopod (Fig. 2C–1). In most instances the proximal spreading of birefringence is more rapid than its distal advance toward the pseudopodial tip. The birefringence does not extend into the hyaline areas that form around the attachment point. As can be seen in Figs. 4 and 5, the birefringence observed in attached pseudopodia can be resolved into an oriented array of birefringent fibrils, each approximately 1 μ in diameter, which extend through the pseudopod and terminate in the attachment area. Upon retraction, birefringence typically fades, but sometimes a rapid reversal of contrast was observed just at the end of the retraction phase. Owing to the rapidity of this phenomenon, it is not known whether there is a reversal in the sign of birefringence.

The fibrillar arrays observed in polarized light are clearly visible as refractile fibrils when viewed through the differential interference microscope (Figs. 6–8). The narrow depth of field characteristic of this optical system at high numerical aperture permitted a determination of fibril location by focusing through the pseudopod. The fibrils appear to lie in the ectoplasm just beneath the cell membrane. Since both the birefringent and the refractile fibrils are evident only after attachment of the pseudopod to the substratum and since they disappear in a similar manner, it is assumed that
they are the same fibrils. In those cases where the plasmalemma appeared folded or rippled, the fibrils did not follow the membrane contour but extended through the pseudopod in a straight course parallel to other fibrils and to the long axis of the pseudopod.

**Syneresis**

As contraction proceeds, drawing the shelled cell body toward the attachment point, numerous hyaline blebs, suggestive of syneresis, appear on the pseudopod surface (Fig. 3). Image duplication interference microscopy (Gahm, 1962, 1963) has shown that the hyaline blebs contain fluid of lower refractive index than the ectoplasm or endoplasm. The blebs are not restricted to any particular region of the pseudopod, but appear all along the surface. The blebs often provide a site for the extension of new pseudopodia which display a morphological and physiological pattern indistinguishable from pseudopodia originating from the cell body. Both types of pseudopodia are similar in regard to size, shape, cytoplasmic streaming, and contractility.

**Behaviour of excised pseudopodia**

The locomotor capacity of pseudopodia isolated from the cell body was evaluated by excising and observing various size fragments. Regardless of how far distally the separation was made, the isolated fragments continued to move for variable periods up to several hours. The fragments displayed both cytoplasmic streaming and consistent translatory movement. Unlike the intact organism, small pseudopodial fragments appeared to be attached along most of their length and moved in a manner more closely resembling the naked amoebae. When larger pieces were obtained by removing the test and excising pieces of the cell body, the fragments assumed a shape similar to the intact organism; that is, pseudopodia extended from a central cytoplasmic body. *Difflugia* have been observed to approach and capture an isolated fragment and subsequently reassimilate this piece into one of their extended pseudopodia.

**Fibril ultrastructure**

The birefringence observed in attached pseudopodia by polarized-light microscopy suggested fibrillar organization at the electron-microscopic level.

Electron-microscopic examination of pseudopodia of *D. corona* revealed 1-μ bundles of microfilaments 55–75 Å in diameter. Fig. 9 represents a section of the cell body demonstrating the nature of the ground cytoplasm and several cytoplasmic components after fixation by the methods described. A layer of endoplasmic reticulum appeared closely aligned along the cytoplasmic side of the nuclear membrane. Several types of vacuoles were present, some of which appeared empty while others contained electron-dense material. The mitochondria displayed a pattern of convoluted tubules commonly found in many protists. On several occasions vacuoles were seen that contained a crystalline inclusion: the nature and function of these structures is unknown. Electron micrographs of attached pseudopodia (Fig. 10) demonstrate bundles of microfilaments in the ground cytoplasm. Some microfilaments diverge from the main bundle and can be seen bending around several cytoplasmic components.
A. Wohlman and R. D. Allen

(Fig. 10, arrow). In this same electron micrograph one can see the numerous vacuoles that lie adjacent to the microfilament bundle. Although there is a net orientation, the microfilaments are not strictly parallel to one another. Some areas of microfilaments appear 'naked' or 'smooth'; however, in most instances particulate material (or perhaps 'side-bridges') seems to be adhering to the filament core. One has the distinct impression of a periodicity in the arrangement of the particulate material. Microfilaments have also been observed both as randomly oriented and dispersed cytoplasmic components, and as aligned filaments in the ectoplasm adjacent to the plasmalemma (Fig. 11).

DISCUSSION

Our knowledge of, and viewpoints toward, amoeboid movement have been greatly influenced by the fact that most workers have chosen to study one of the more abundant giant, naked, free-living, carnivorous amoebae (for example, Amoeba proteus or Chaos carolinensis). Difflugia extends cylindrical pseudopodia in a surprisingly similar manner, but that is where the similarity ceases. Unlike the afore-mentioned naked amoebae, Difflugia forcibly retracts its pseudopodia while they are attached to the substratum, with the result that the heavy shell is dragged forward.

What makes amoeboid movement in Difflugia especially interesting is that a special array of fibrils is rapidly laid down before pseudopod retraction occurs. The formation of this array between the cell body and a forward pseudopodial attachment point takes only a few seconds, and the array itself may be more highly birefringent and more impressive in extent than the mitotic spindle of a marine invertebrate egg. During pseudopod retraction the array fades and disappears.

Forcible pseudopod retraction is not a well-established general feature of amoeboid movement. Although many have claimed that contraction of the tail is the driving force (by hydraulic pressure) for the formation of new pseudopods (see Goldacre, 1952), the evidence in favour of this idea is not convincing, and the behavioural evidence against it is considerable. In the case of Difflugia, forcible pseudopod retraction occurs without any doubt. It is interesting to note, however, that retraction is not necessarily temporally correlated with extension of a new pseudopod, as would be expected if the latter formed as the result of an increased internal hydraulic pressure.

Forcible retraction of pseudopodia seems to be a general feature of many if not all testaceans, especially those with the heavier tests, and probably occurs also in some of the more obscure species of small free-living amoebae. Forcible pseudopod retraction has also been observed in sea-urchin gastrula mesenchyme cells (Gustafson & Wolpert, 1963; Gustafson, 1964). One might guess that this type of cell movement is a more common feature of embryogenesis, histogenesis, and regeneration than is commonly recognized. Unfortunately, many of the 'working pseudopodia' in embryos have so far been quite inaccessible to study. Presumably Difflugia serves as an important model system for the study of this type of pseudopod.

The term 'forcible retraction' implies that something contracts. Since the time of Engelmann (1879) it has been assumed that contraction can occur only in an aniso-
Pseudopods of Diffugia

tropic material. Optical anisotropy (especially positive birefringence) has been observed in nearly every structure known or suspected to be contractile (myofibrils, cilia, flagella, reticulopodia, axopodia etc; see Schmidt (1937) for review). Recent studies from this laboratory (Allen, Francis & Nakajima, 1965) established that pseudopodia of Chaos carolinensis are normally weakly birefringent \((B = 2 \times 10^{-6})\), as if they contained either a very few protein fibrils or consisted of a weakly organized gel. The same is true of newly forming pseudopodia of Diffugia: the weakly positive axial birefringence is barely detectable during pseudopod extension except during sporadic flow, when flashes of birefringence are observed. This pattern of birefringence changes is strikingly similar to that reported in Chaos by Allen et al. (1965), and it is tempting to attribute the phenomenon to the same cause in both cases: a build-up in tension between the postulated site of contraction at the pseudopodial tip (Allen, 1961b) and a region of resistance more posteriorly located.

The birefringent material that forms into arrays of fibrils just before contraction is particularly noteworthy, for the timing of its appearance in the pseudopod retraction cycle strongly suggests a contractile role. Engelmann, had he found these structures, might have claimed them as his postulated ‘Inotagmen’, or contractile elements. While the behavioural evidence is suggestive of a contractile role for these fibrils, we cannot prove this from either the light- or the electron-microscopic evidence so far. The diminution of birefringence during pseudopod retraction could be due either to destruction or disorientation of the fibrillar material. The electron microscope reveals only seemingly static bundles of 55–75 Å microfilaments, without any indication of how these elements might participate in a contractile event. We cannot exclude the possibility that the microfilaments constitute only a part of the contractile machinery, the rest of which has not been preserved.

The manner in which the fibrillar material appears and certain aspects of pseudopod behaviour allow us to infer something of the mechanism by which the fibrillar material is laid down. New pseudopods are almost isotropic, indicating that if the subunits of the fibrillar material are present, they are randomly oriented. During the late stages in pseudopod extension, just before attachment, pseudopodia typically wave about, almost like the tentacles of an octopus, before settling on the substratum. This may be due to the development of unequal tension along different portions of the newly formed ectoplasmic tube. An adhesive pseudopod, making contact with the substratum, might be subject to internal forces that would normally cause it to bend, but be unable to bend by virtue of its recent attachment to the substratum. This in turn would result in the development of stress in the ectoplasmic tube between the attachment point and cell body. Orientation within the ectoplasmic tube gel caused by the stress might serve to orient further polymerization of fibrils. It is regrettable that more has not been learned from electron microscopy of this fibrillar material, its formation and role in contraction. It is hoped that better fixation media can be found.

Brief mention deserves to be made of the ability of non-nucleated pseudopod fragments of Diffugia to move in a coordinated and organized manner (see also Kepner & Reynolds, 1923). This is a somewhat different situation from that which exists
in *A. proteus*, where non-nucleate fragments lose the power of successful locomotion within a few minutes after enucleation. The retention of the ability of pseudopod fragments to move independently leaves little doubt that the pseudopods themselves contain the necessary motile machinery.

We are grateful to Professor L. I. Rebhun for many suggestions concerning fixation for electron microscopy. This work was supported by a research grant from the National Institute of General Medical Science.

REFERENCES


GOLDACRE, R. J. (1952). The folding and unfolding of protein molecules as a basis of osmotic work. *Int. Rev. Cytol.* 1, 135–164.


Pseudopods of Difflugia


(Received 26 April 1967—Revised 1 August 1967)
The scale on light micrographs represents 10 μ per division.
Fig. 1A-H. Retraction of a pseudopod while the endoplasm continues to flow in a distal direction. Nomarski differential interference contrast optics. Frames at 2-sec intervals.
Fig. 2A–I. The development of birefringence at the attachment point and subsequent spreading away from this point. Frames at 50-sec intervals.

A. WOHLMAN AND R. D. ALLEN
Fig. 3A–L. Syneretic blebs forming on the surface of a retracting pseudopod and subsequent development of pseudopodia from the sites of bleb formation. Nomarski differential interference contrast optics. Frames at 30-sec intervals. The image obtained with the Nomarski system is an 'optical shadow-casting effect' caused by interference contrast generated as a function of the gradient of optical path difference.
Figs. 4, 5. Birefringent fibrils in attached pseudopodia. Zeiss polarized-light optics. This pair of micrographs shows the reversal of contrast at opposite compensator bias compensation settings.

Fig. 6. Refractile fibrils in attached pseudopodia. Nomarski differential interference contrast optics.

Figs. 7, 8. Refractile fibrils terminating at the attachment area. Fig. 8 shows the orientation of the organism which is suspended from the underside of a coverslip. Nomarski differential interference contrast optics.

A. WOHLMAN AND R. D. ALLEN
Fig. 9. Electron micrograph showing area within the cell body (c, chromatin; cv, crystal-containing vacuoles; db, dark bodies (probably lipoids); er, endoplasmic reticulum; m, mitochondria; n, nucleus; nm, nuclear membrane; r, ribosomes). × 24,000.

A. WOHLMAN AND R. D. ALLEN
Fig. 10. Aligned, densely packed microfilaments. The arrow points to several microfilaments bending around cytoplasmic inclusions. $\times 40000$.

Fig. 11. Microfilaments ($mf$) in the ectoplasm just adjacent to the plasmalemma. $\times 36000$.