FIBRILLAR STRUCTURES IN THE CYTOPLASM OF *CHAOS CHAOS*

VIVIANNE T. NACHMIAS. From the Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia

INTRODUCTION

Although a number of studies of the fine structure of fixed amebae have appeared (Pappas, 1959; Mercer, 1959; Roth, 1960; Brandt and Pappas, 1962), fibrillar structures in the cytoplasm have not been reported. This is surprising in view of the fact that several current theories of ameboid movement postulate the existence of contractile and hence presumably fibrillar material. This discrepancy between what is thought to exist in living amebae and what has so far been found in fixed specimens has recently been emphasized (Goldacre, 1961; Pitelka, 1963).

Furthermore, there is mounting evidence from other types of studies that contractile structures exist in amebae. To cite only a few examples, Kriszat (1949) and Goldacre and Lorch (1950) found that adenosine triphosphate produced characteristic changes in the cytoplasmic consistency of amebae. Simard-Duquesne and Couillard (1962) reported that a glycerinated ameba preparation contracted upon addition of adenosine triphosphate and magnesium ion. Most recently, Wolpert, Thompson, and O'Neill (1964) have provided electron microscope evidence for fibrillar material in preparations of centrifuged ameba cytoplasm induced to gelate by addition of adenosine triphosphate.

The present communication reports the finding of extensive areas of fibrillar material in sections of amebae fixed after exposure to Alcian Blue, a dye which binds irreversibly to the cell surface and induces pinocytosis at room temperature.

MATERIALS AND METHODS

On initiating this study, it seemed possible that previous failures to observe fibrils in fixed amebae might be due to special characteristics of fibrils in amebae or to the impermeability of the large, free-living species usually used. First, fibrils in amebae would be expected to be labile structures. This seems likely both from simple observations of the dramatic variations in shape that are easily produced by shaking, chilling, etc., and from work on changes in cytoplasmic consistency when temperature, ionic strength, pH, or pressure are varied (Mast and Prosser, 1932; Landau, Zimmerman, and Marsland, 1954). To increase the probability of finding labile fibrils, amebae were exposed to a dye that induces pinocytosis. This was done because it has been suggested that pinocytosis involves the attachment of the plasmalemma to the underlying gel (Marshall, Schumaker, and Brandt, 1959) and an increase in gelated regions (Chapman-Andresen, 1962). It was also decided to depart from conventional alkaline fixation, and to follow the methods that Roth and co-workers (Roth and Jenkins, 1962; Roth and Daniels, 1962) found successful in preserving the mitotic spindle, another labile fibrillar structure. Claude (1962) also reported that certain structures are not preserved by alkaline osmium solutions. Hence unbuffered osmium tetroxide (pH about 6) was used. Calcium ions were added since it has been found that divalent ions stabilize the structure of the mitotic spindle (Roth and Daniels, 1962). Calcium ions have been added to osmium fixatives in previous studies of amebae, but at alkaline pH (Pappas, 1959; Brandt and Pappas, 1962).

Secondly, the plasmalemma of *Chaos chaos* is remarkably impermeable to water in the living state (Belda, 1943; Løvtrup and Pigon, 1951). It might well delay the entry of a slowly penetrating fixative like osmium tetroxide. Indeed, one can frequently see signs of movement in amebae for a few minutes after the addition of this fixative. For this reason, the plasmalemmas of some of the amebae in this study were gently torn with sable hairs, immediately before adding fixative, to allow direct contact of fixative and cytoplasm. Control amebae treated in this way

after exposure to dye, but not fixed, frequently sealed the break and eventually moved about normally.

CULTURE: *Chaos chaos* (*Pelomyxa carolinensis*) was cultured in glass according to the method of Marshall (in preparation). The medium consisted of 5×10^{-4} M $CaCl_2$, 5×10^{-5} M $MgSO_4$, 1.13×10^{-4} M K_2HPO_4, with a resultant pH of 6.8–7.0.[1] Amebae were fed each day with a suspension of *Paramecium aurelium*. On the morning of an experiment, before feeding, medium-sized individuals were removed with braking pipettes (Holter, 1943) to embryological watch glasses.

PREPARATION OF AMEBAE: Amebae were rounded up by chilling and gently shaking in cold (5°C) medium. They were stored at 5° for about an hour, then washed in cold ion-free water. They were transferred to dye solution also at 5°C. Alcian Blue was used as a 0.05 per cent solution in ion-free water. This concentration was found by Chapman-Andresen (1962) to induce pinocytosis at room temperature.[2] Amebae were exposed to the dye solution with continuous gentle shaking. At 5°C, cytoplasmic movements are inhibited, and uptake of membrane does not occur, but the dye is strongly and irreversibly bound to the cell coat. After 2 minutes, the amebae were thoroughly rinsed with cold ion-free water, and removed to a drop of medium at room temperature (20–25°C). Control experiments showed that dye-treated amebae left undisturbed put out a new pseudopod within 10 minutes. The experimental cells were, therefore, fixed about 5 minutes after removal to room temperature, *i.e.* shortly before formation of a new pseudopod. A total of 12 amebae were examined, 3 of which were left intact, while the plasmalemmas were torn in 9. Dishes and pipettes used during or after exposure of the cells to dye were silicone treated, since exposure to the dye made the cells very sticky and introduced the chance of accidental damage if this precaution was omitted.

FIXATION: 2 per cent unbuffered osmium tetroxide was used at room temperature and contained 0.01 M $CaCl_2$. Fixation was carried out for 7 minutes followed by brief rinsing in ion-free water, dehydration in ethanol-water mixtures, and embedding in Araldite. Thin sections were cut with a diamond knife and collected on uncoated grids. For staining, 2 per cent phosphotungstic acid in 95 per cent alcohol was used. Sections were examined in an RCA EMU 3C.

[1] This solution will be referred to as "medium" in this paper.

[2] Alcian Blue was obtained from the Hartman-Leddon Co., Philadelphia, Item # 109.

RESULTS

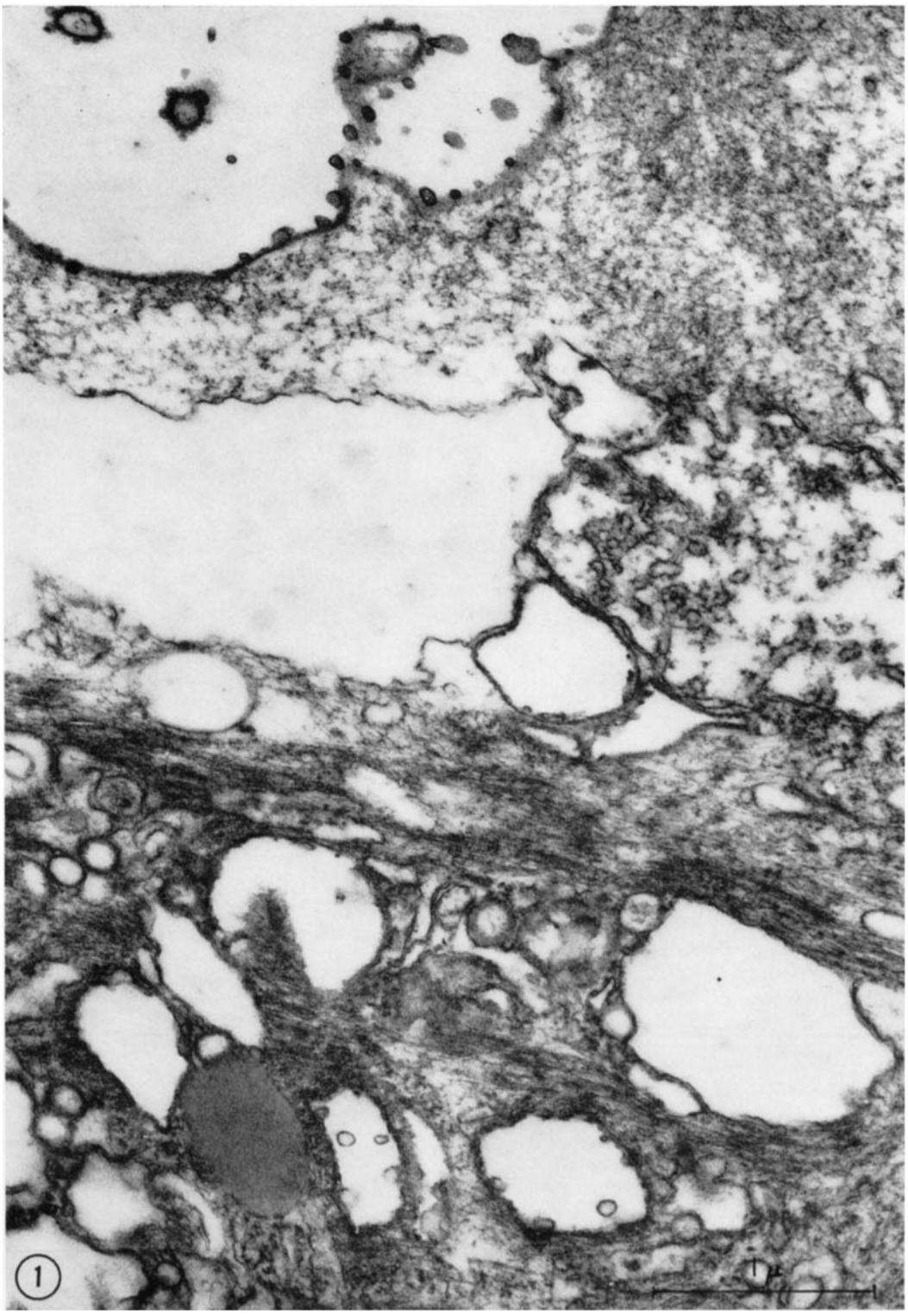
Fig. 1 shows an example of fibrillar structure seen in an ameba undisturbed before fixation. The surface hairs or fringes usually seen are replaced here by clumps representing the interaction of dye with the hairs. The clumping is not due to the fixative used but to the action of the dye, as it can be seen in isolated membranes picked up directly onto collodion-coated grids. Immediately beneath the plasmalemma can be seen a region composed of granules and fine fibrils arranged in network fashion. Deeper in the cytoplasm, the fibrils are arranged in bands running roughly parallel to the surface of the ameba. Higher magnifications have shown the fibrils to be of the order of 70 to 80 A in diameter.

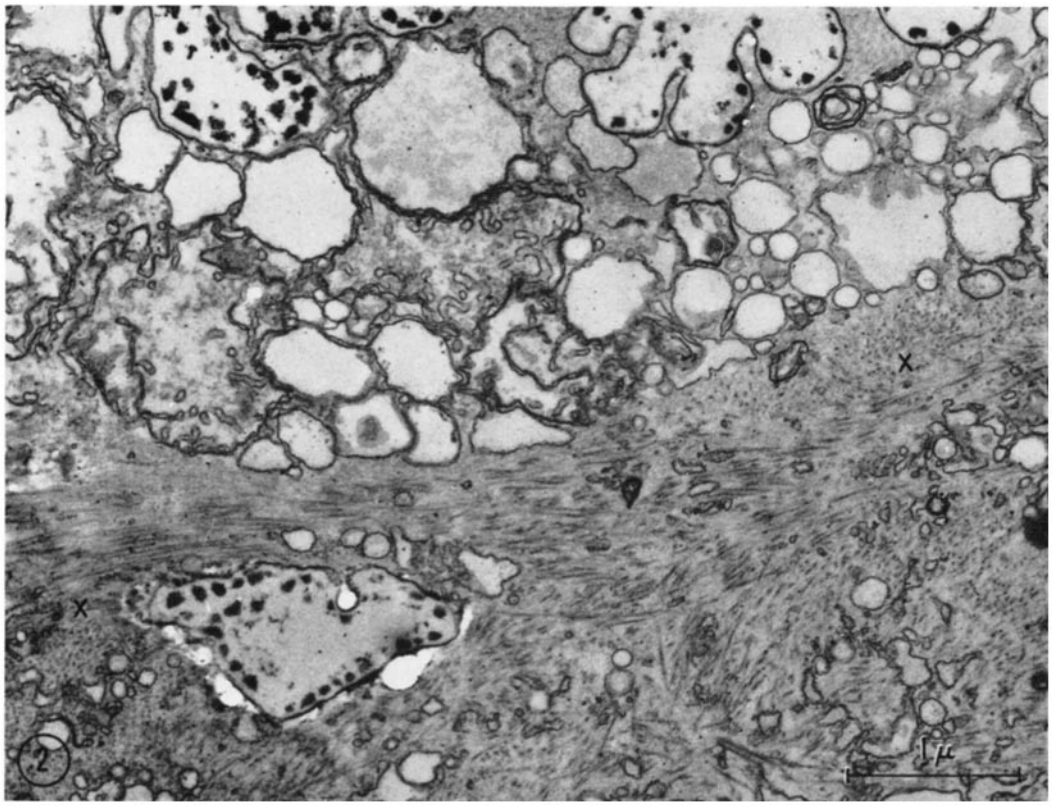
Fig. 2 shows a similar view of an ameba whose plasmalemma was torn before fixation. The network region is less well shown in this figure, but was usually present with equal or greater frequency than in undisturbed amebae. The parallel fibrils lying more deeply in the cytoplasm are here of greater diameter than in Fig. 1. Higher magnifications have shown them to be about 150 A in diameter and up to 0.5 μ in length.

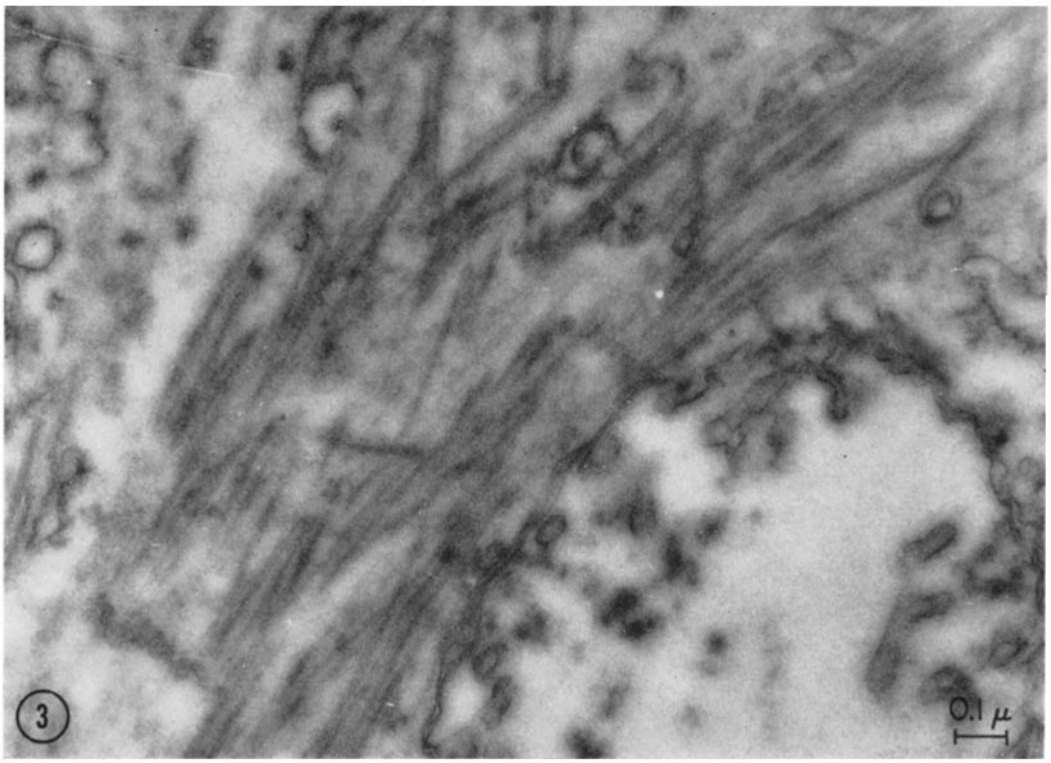
Fig. 3 is from an ameba treated as in Fig. 2, to show the thicker fibrils at higher magnification.

In all the amebae the fibrillar regions had to be searched for, *i.e.*, their distribution was limited. A consistent finding was that fibrils were seen most frequently in cytoplasm subjacent to convoluted membrane. The only consistent difference between intact amebae and those whose plasmalemmas were torn was that large zones of thicker fibrils were seen more often and in larger numbers in amebae whose plasmalemmas had been torn.

FIGURE 1 Surface and cytoplasm of *Chaos chaos* after exposure to Alcian Blue. Cells were left undisturbed before fixation. Fixation: 2 per cent unbuffered osmium tetroxide containing 0.01 M $CaCl_2$. Stained with phosphotungstic acid. Note the dense clumps along the surface of the cell (at top), representing the interaction of dye and surface fringes. Also note that the network region is subjacent to the surface while fibrils oriented parallel to one another occur deeper in the cytoplasm ×34,500.







However, such zones were also found in the undisturbed cells.

DISCUSSION

It seems likely that the fibrillar structures demonstrated here are connected with cytoplasmic movements, and experiments are in progress to explore this. It does not seem likely that the fibrils are artifacts of fixation, for the following reasons: (1) The dimensions and arrangements of the fibrils are very constant within the limitation mentioned above. (2) The two types of fibrils found correspond quite closely in size to those seen by Wolpert, Thompson, and O'Neill (1964) in ameba cytoplasm extracts after gelation with adenosine triphosphate. (3) Amebae treated with Alcian Blue have recently been homogenized, and fibrils of the dimensions found in fixed material can be seen in negatively stained preparations without any fixation (Nachmias, unpublished).

It is curious, however, that the thick fibrils are found so much more frequently in amebae whose plasmalemmas have been torn. The criticism may be leveled that the thick fibrils are, in some way, the result of the interaction of the calcium ion (present at high concentration) with cytoplasm. The other possibility is that the impermeability of the plasmalemma is indeed a factor, and that the thick fibrils are labile enough to be inadequately fixed by osmium tetroxide entering slowly through an intact plasmalemma. Experiments with other fixatives are now planned to try to settle this point.

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FIGURE 2 Ameba treated with Alcian Blue, but plasmalemma torn immediately before fixation. Fixed and stained as in Fig. 1. Note that the fibrils here are of larger diameter than those in Fig. 1. Also note cross-section of fibrils in regions marked with an *x*. ×22,500.

FIGURE 3 Ameba treated as in Fig. 2. The thicker fibrils at higher magnification. No stain used. ×68,500.

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