

FURTHER ELECTRON MICROSCOPE STUDIES ON FIBRILLAR ORGANIZATION OF THE GROUND CYTOPLASM OF *CHAOS CHAOS*

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ABSTRACT

Further evidence for fibrillar organization of the ground cytoplasm of *Chaos chaos* is presented. Fixations with osmium tetroxide at pH 6 or 8 and with glutaraldehyde at pH 6 or 7 were used on two preparations: (a) single actively streaming cells; (b) prechilled cells treated with 0.05% Alcian blue in the cold and returned to room temperature for 5–10 min. In addition, a 50,000 g pellet of homogenized cells was examined after fixation with glutaraldehyde-formaldehyde alone. In sections from actively streaming cells considerable numbers of filaments were observed in the uroid regions after glutaraldehyde fixation, whereas only traces of filaments were seen after osmium tetroxide fixation at either pH 6 or 8. Microtubules were not seen. In sections from dye-treated cells, filaments (4–6 m μ) and fibrils (12–15 m μ) were found with all three fixatives. The 50,000 g pellet was heterogeneous but contained both clumps of fibrils and single thick fibrils like those seen in the cytoplasm of dye-treated cells. Many fibrils of the same dimensions (12–15 m μ wide, 0.5 μ long) were also seen in the supernatant above the pellet. Negative staining showed that some fibrils separated into at least three strands of 4–6 m μ filaments.

INTRODUCTION

In this paper we propose to strengthen the evidence for the existence of filaments and fibrils in the cytoplasm of the giant ameba, *Chaos chaos*.

Interest in the phenomena of cytoplasmic streaming, amoeboid movement, and more generally, all types of primitive motility has increased owing to recent advances along three different lines of work. First, fine structure studies made since the introduction of aldehyde fixatives for electron microscopy (Sabatini et al., 1963) have shown a suggestive relationship between the occurrence of microtubules and streaming in some plant and animal cells (Ledbetter and Porter, 1963; Rudzinska, 1965; Porter, 1966).

Second, remarkable advances have been made in the understanding of contraction in vertebrate striated muscle. Of these, the most relevant to

primitive motility has been the introduction of the sliding filament model of contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954; Huxley, 1960, 1963). The essential feature of the model is that muscle contraction depends on a cyclic interaction between the thick and thin filaments which are composed, in an ordered way, of at least two different proteins oriented with appropriate polarity and remarkable precision. Many elegant studies have been made to test the model for vertebrate striated muscle, and its general features are accepted beyond reasonable doubt. The idea that a sliding force could occur between a gel phase and a sol phase during some types of streaming has been suggested (Loewy, 1949; Jahn and Rinaldi, 1959), but there is still no critical evidence for such a process.

Third, biochemical studies on the slime mold *Physarum* which had already shown the existence of an actomyosin-like protein (Loewy, 1952; Kamiya, 1959; Nakajima, 1960) have recently resulted in the discovery (Hatano et al., 1967) of two types of actin polymer. One type is remarkably similar to fibrous actin from muscle; the other, which forms in the presence of magnesium, is apparently globular or amorphous and has a much lower specific viscosity than the former type, while retaining ATPase activity.

These major advances open up interesting possibilities for the understanding of cytoplasmic streaming and ameboid movement. However, our knowledge of how the structures that exist in streaming systems are related to the physiological events is still very primitive. Although the freshwater amebae are not well-suited for biochemical work, their size and ease of manipulation make them useful for this kind of approach to the problem. At present, neither in the freshwater amebae nor in *Physarum* have there been found either microtubules (except during mitosis: Roth and Daniels, 1962) or thick filaments analogous to the characteristic filaments of striated muscle. The filamentous structures that do exist still pose fixation problems. In early electron microscope studies of amebae, no structural elements that might provide a basis for contractility were seen (Mercer, 1959; Roth, 1960; Brandt and Pappas, 1962). Since then, several studies have demonstrated filaments or fibrils in amebae after pretreatments of various kinds: incubation with surface-bound dye (Nachmias, 1964) or with enzymes (Komnick and Wohlfarth-Bottermann, 1965), or injection of fixative (Bhowmick and Wohlfarth-Bottermann, 1966).

In this paper we show that fibrils also occur in untreated cells. However, they seem to be more prevalent in dye-treated cells. We also show that fibrils occur in a pellet fraction from homogenized, dye-treated amebae. Two types of filaments, thin (4–6 $m\mu$) filaments and thick (12–15 $m\mu$) fibrils,¹ can be found after either osmium tetroxide or glutaraldehyde fixation. The relationship between these two types of filaments may be important for the understanding of streaming in amebae.

¹The term "fibril" is used for the thick form because this form is of limited length and also because the term avoids confusion with the "thick filament" of striated muscle.

MATERIALS AND METHODS

Culture

Chaos chaos was grown, as previously described, on *Paramecium aurelium* (Nachmias, 1966).

Preparation of Untreated Cells

2-day starved, vigorously streaming specimens were transferred with braking pipettes to small culture dishes in approximately 0.05 ml of culture fluid. Dishes were on the stage of a binocular microscope illuminated from below. Vigorous streaming was maintained by shading the anterior part of the ameba. The animals are negatively phototactic and continue to stream forward into the darker region.

Alcian Blue Treatment and Pellet Preparation

Dye treatment for 10–20 cells was as described previously (Nachmias, 1964, 1966). Upon return to room temperature, dye-treated cells resume streaming after 10–15 min. Usually, cells are monopodal at first, but otherwise streaming appears normal. Dye-coated surface accumulates at the uroid (Nachmias, 1966). For this study, individual cells were fixed just as visible streaming resumed. For pellet preparation, 0.2–0.5 ml of packed, 2-day starved cells were treated with 10 ml of 0.05% Alcian blue in the cold and were washed four times with culture fluid; the cell pack was then returned to room temperature for 3–5 min, until the internal temperature of the cell pack was about 16–18°C. At this time cells have not resumed visible streaming. The cells were then homogenized with a No. 25 needle, or in a 2 ml all-glass homogenizer for 2 min after the addition of 10 μ moles of Tris-ATP (pH 7) per milliliter of homogenate to produce a final concentration of 10 mM Tris-ATP.

The homogenate was centrifuged at 1700 g for 10 min, and the supernatant was respun at 50,000 g for 15 min at 4°C. The pellet obtained was fixed in a mixture of freshly prepared formaldehyde and glutaraldehyde (Karnovsky, 1965) for 1 hr, dehydrated in alcohol, and embedded in Araldite. Thin sections were made and stained with phosphotungstic acid. Drops of the supernatant above the pellet were used for negative staining which was done according to Huxley's (1963) method with 2% uranyl acetate, pH 4.3.

Chemicals

Alcian blue 8 GX, purchased from Allied Chemical Corp. (Morristown, N. J.), was kept as 0.1% stock solution and was filtered before use. Tris-adenosine triphosphate was a Sigma Chemical Co. (St. Louis, Mo.) product; the stock solution (titrated to pH 7 with NaOH) was stored frozen until used. Glutaraldehyde was obtained from two sources: Union

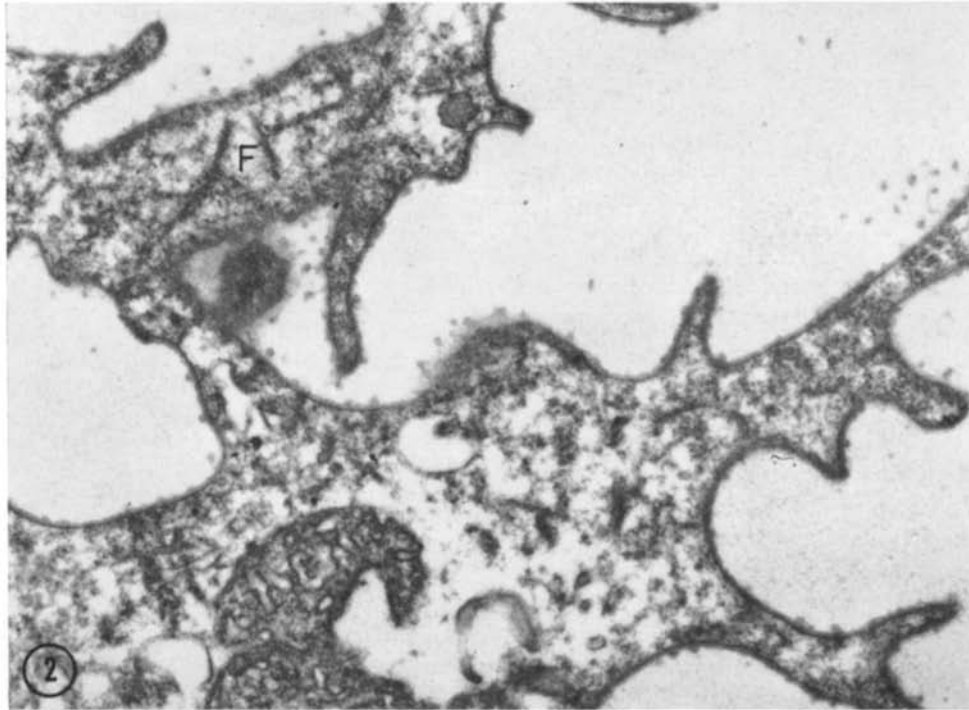
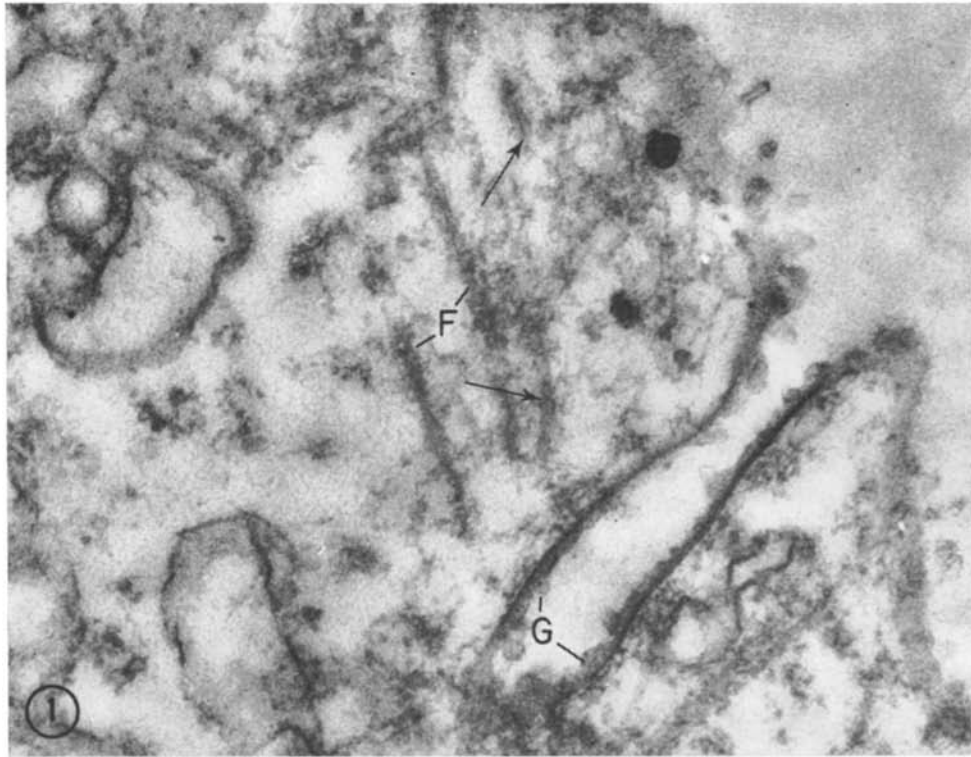


FIGURE 1 Uroid region from *Chaos chaos* fixed with 2% osmium tetroxide in distilled water containing 10 mM CaCl₂. *F* and arrows, traces of fibrillar material; *G*, surface coat globules. Compare with Figs. 2 and 3. $\times 68,000$.

FIGURE 2 Uroid region from *Chaos chaos* fixed with 2.5% glutaraldehyde buffered at pH 7 with 0.2 M phosphate and postfixed with osmium tetroxide. Minimal distortion during fixation. *F*, thick fibrils. $\times 15,000$.

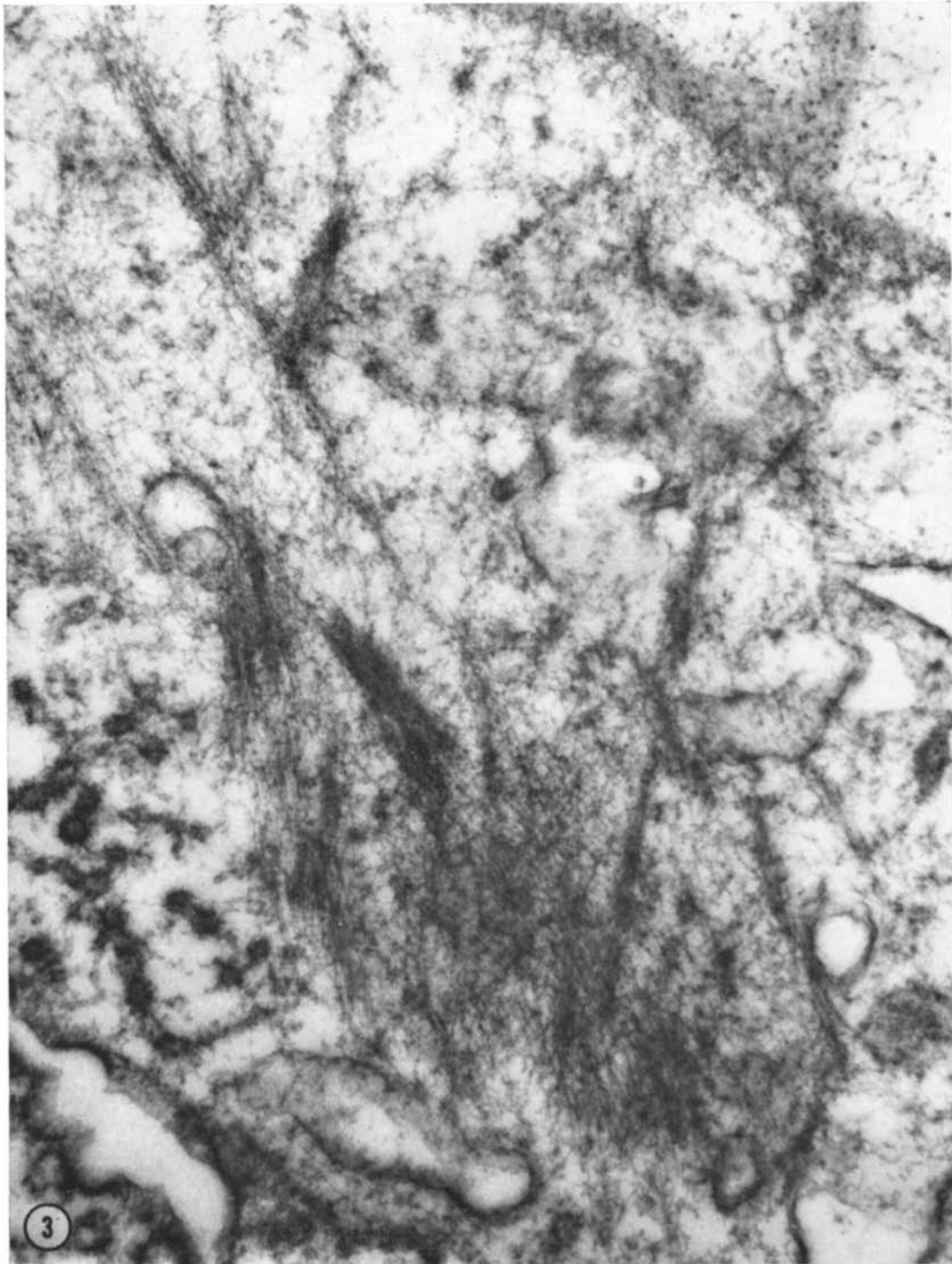


FIGURE 3 Same as Fig. 2. Surface of cell at upper right corner. $\times 50,000$.

Carbide Corporation (New York), 25%; and Fisher biological grade (Fisher Scientific Company, Pittsburgh, Pa.), 50%. In early experiments the former glutaraldehyde was used; in some of the later experiments, the glutaric acid present was precipitated with barium carbonate. This procedure resulted in a rise in pH from the initial 3.4 to 6.7, but no difference was observed in the amount of distortion of cells during fixation after this procedure.

Fixation

For single cells, 0.25 ml of one of the following fixatives was added while the organism was being observed under a binocular microscope. (a) 2.5% glutaraldehyde buffered with 0.2 M phosphate buffer to pH 6 or 7. In later experiments, which employed pH 6-7 glutaraldehyde after precipitation of glutaric acid, culture fluid was also used as a weak buffer. (b) 1% osmium tetroxide, buffered to pH 8 with Veronal-acetate (Pappas, 1959). (c) 2% unbuffered osmium tetroxide (Claude, 1961), pH 6-6.5, with the addition of 1 or 10 mM calcium chloride. Cells were fixed for 10 or 20 min. A cursory sketch of each cell was made before fixation, and those cells exhibiting gross distortions of shape were discarded. Distortion was common with glutaraldehyde fixation, but was very rare with osmium tetroxide fixation (Table I). Glutaraldehyde-fixed cells were washed in a large

excess of ion-free water, then postfixed in 2% osmium tetroxide for 10 or 20 min. The duration of fixation as recorded in Table I was the duration of visible streaming at a magnification of 60 after the addition of fixative. The estimates of two observers agreed very well.

After fixation and dehydration in alcohols, the amoebae were embedded in Epon/Araldite. The anterior pseudopodial regions or uroid regions were chosen for examination; the remaining half of the amoeba was cut away. Sections were cut on Servall MT-1 or MT-2 microtomes, mounted on Formvar-coated grids, and stained with phosphotungstic acid or lead. They were examined in an RCA-EMU-3 or a Hitachi HU-11A electron microscope at initial magnifications of 4,000-40,000.

RESULTS

Different Fixations of Uroid Regions

Fig. 1 shows the maximum amount of fibril structures seen after an exhaustive search of tail regions in more than 10 amoebae fixed with either of the two kinds of osmium tetroxide. *F* and arrows mark the region of fibrillar material present in traces; the surface coat is represented by globules (*G*). This figure shows a specimen fixed

TABLE I

Results of a Typical Experiment on Duration of Fixation in Chaos chaos.

Cells in 0.05 ml of culture fluid; 0.25 ml of fixative added at room temperature. Addition of fixative required about 15-20 sec.

Fixation used	pH	Time until streaming stopped	Shape distortion*
		<i>sec</i>	
2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 6 about the same results)	7	120 180	+ back flow +
2.5% glutaraldehyde in culture fluid	6.7	128	++
2.5% glutaraldehyde plus 5 mM EDTA in culture fluid	6.4	123 85 90 110	++ to +++ in all, lysis in 1
2.5% glutaraldehyde in culture fluid plus 15 mM MgCl ₂	6.9	122	++
2.5% glutaraldehyde plus 2.5% dimethyl sulfoxide	7	180-230 (4 trials)	++ to +++ membranes often lift up from cytoplasm
2% unbuffered osmium tetroxide	6.5	0-2 0-2 0	0
1% alkaline osmium tetroxide	8	0-2	0

* 0 to +++.

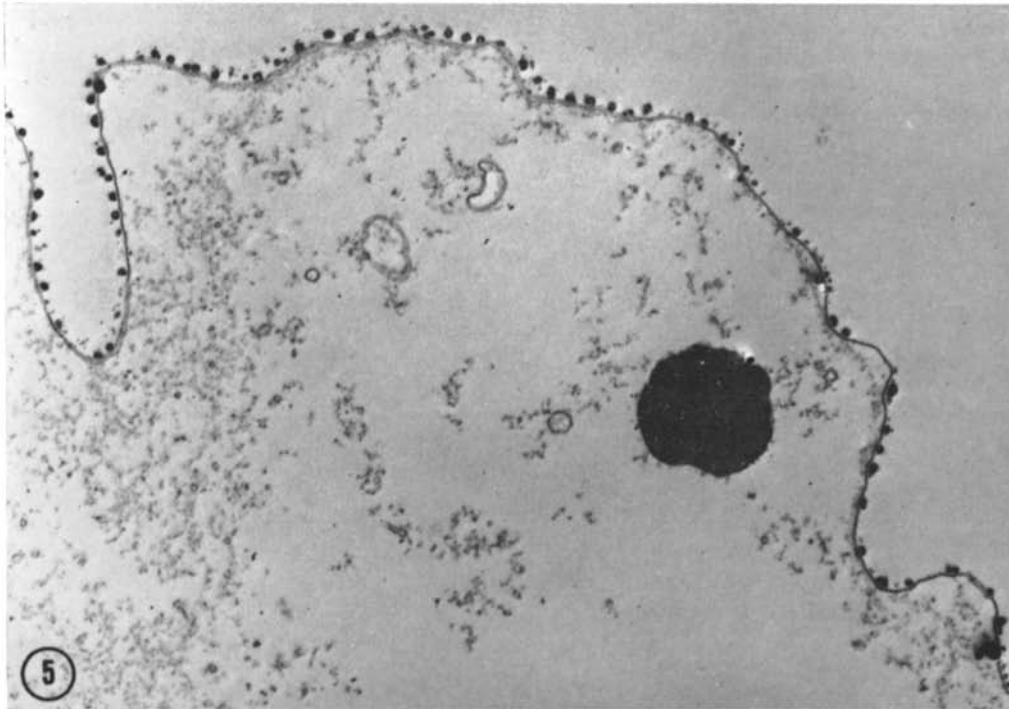
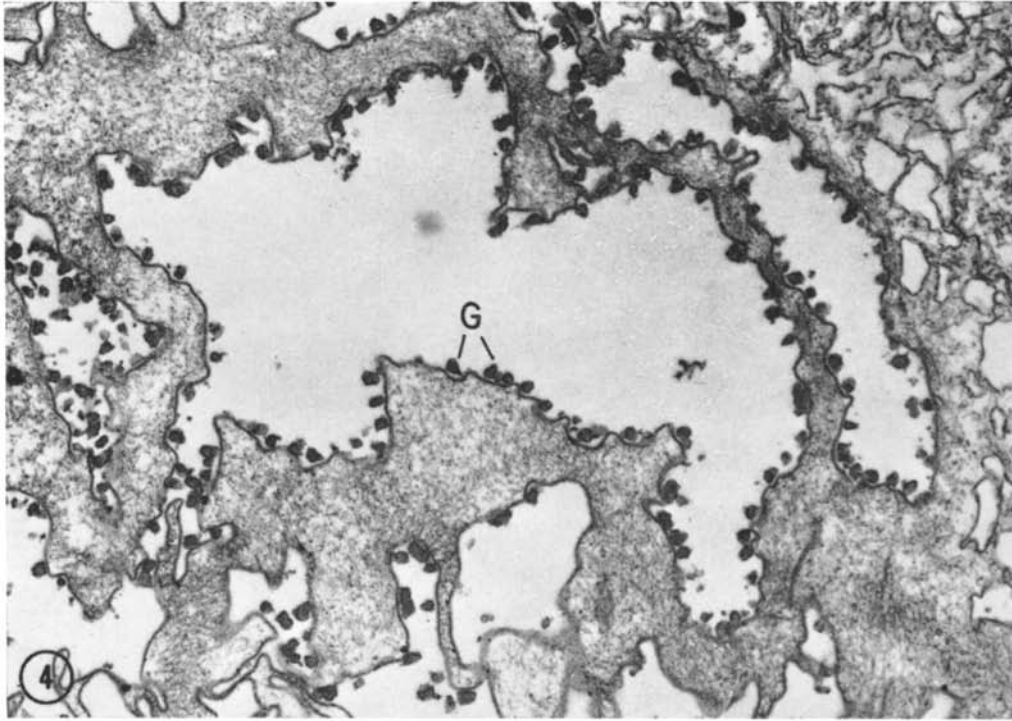


FIGURE 4 Uroid region from *Chaos chaos* fixed when beginning to stream after treatment with Alcian blue. Fixation: 2% osmium tetroxide with 10 mM CaCl₂. G, surface coat globules. Compare with Fig. 5. $\times 13,500$.

FIGURE 5 Pseudopodial region from *Chaos chaos* as in Fig. 4. Same fixation as for Fig. 4. $\times 13,500$.

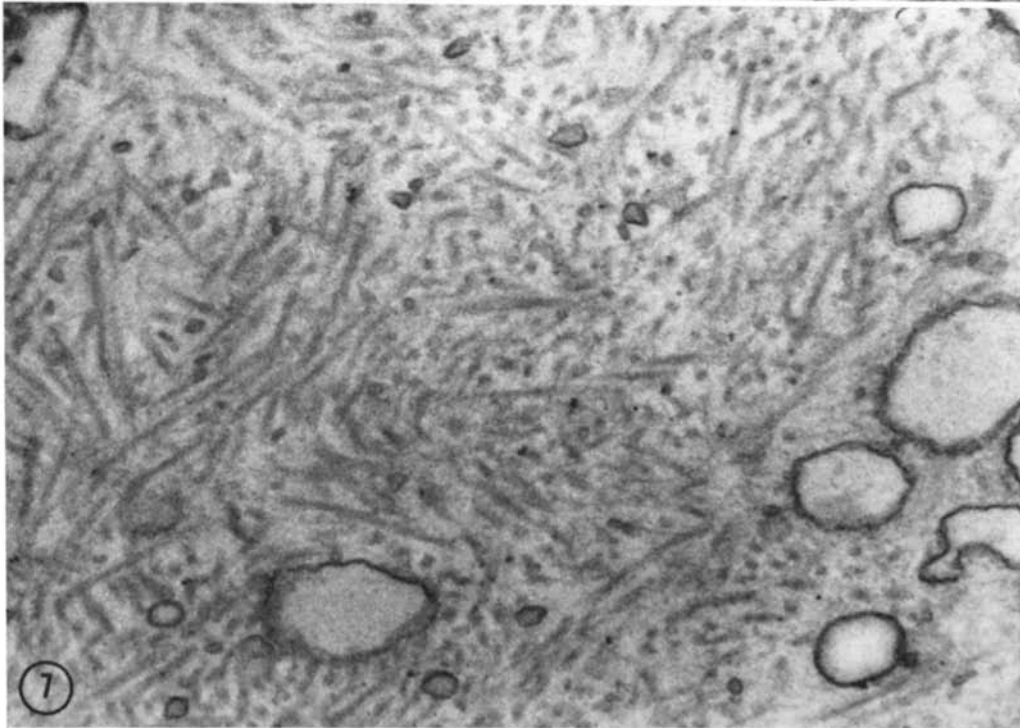
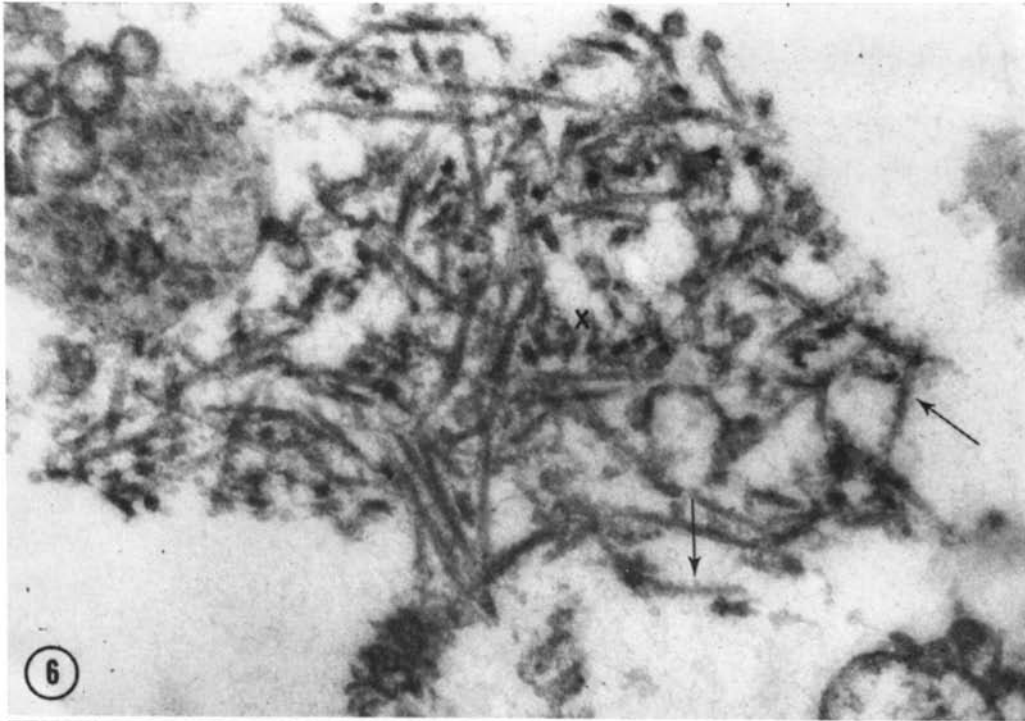


FIGURE 6 Section of pellet to show group of thick fibrils. *X* is placed just above a row of fibrils cut in cross-section. Compare with Fig. 7. $\times 60,000$.

FIGURE 7 Area of *Chaos chaos* after dye treatment to show maximal amount of thick fibrils deep in the cytoplasm. Comparison with Fig. 6 shows that fibrils are similar to those in the pellet. $\times 50,000$.

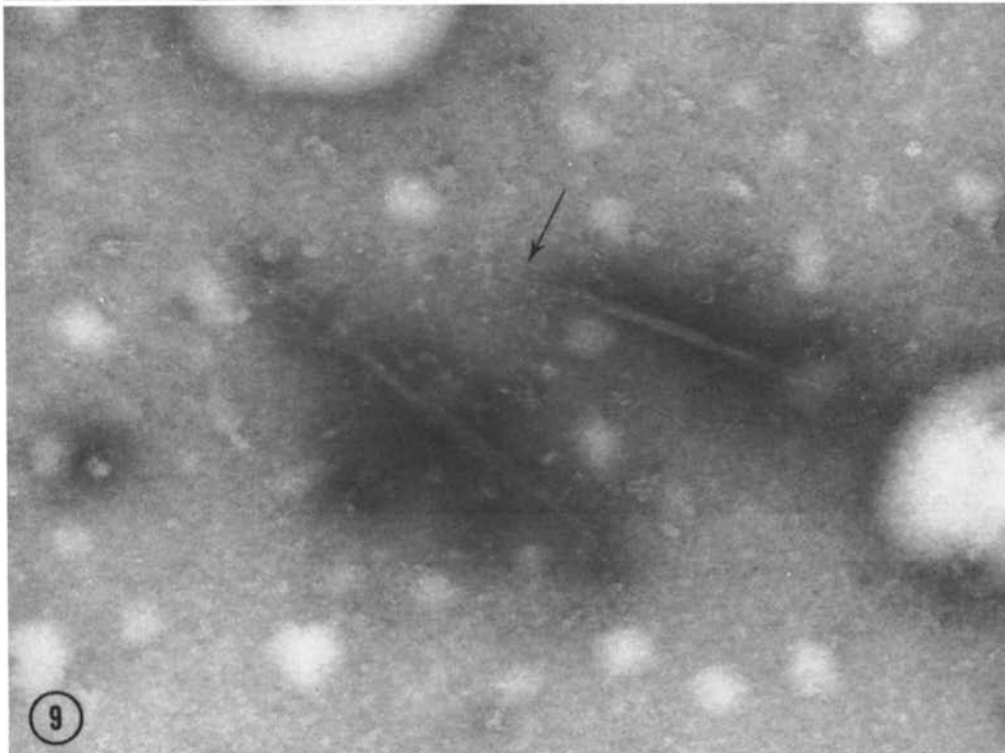
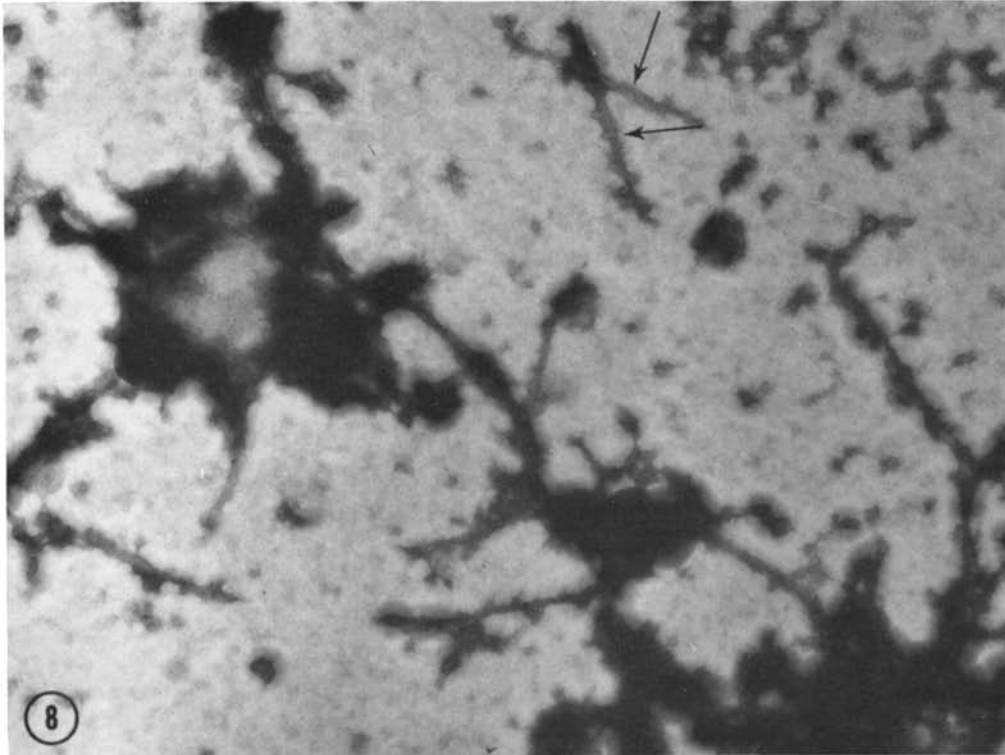


FIGURE 8 Positively stained area in supernatant taken from just above 50,000 *g* pellet. Compare with Fig. 6. Arrows point to two thick fibrils. $\times 60,000$.

FIGURE 9 Negatively stained area of another preparation as in Fig. 8. No fixation. One thick fibril appears to be separated at one end into at least three, probably more, filaments about 4-6 μ wide (arrow). $\times 100,000$.

with the unbuffered osmium tetroxide containing 0.01 M Ca ion; even this much structure was not seen with the alkaline osmium tetroxide. For contrast, Figs. 2 and 3 show the typical appearance of uroid regions after glutaraldehyde fixation (three amebae). Fig. 2, at lower power, shows a small area of thick fibrils (*F*). Fig. 3 shows both networks of thin filaments and areas where thin filaments lie in parallel array.

Differences Between Parts of Cells

As Table I shows, glutaraldehyde always produced *some* form distortion of the cell during fixation. When distortion was minimal in the uroid, back flow from the pseudopodial regions was still obvious. Therefore, pseudopodial regions were not examined after glutaraldehyde fixation. After *osmium tetroxide* fixation, tail regions were characterized by (*a*) highly convoluted plasmalemmas, (*b*) denser ground cytoplasm than in anterior regions, (*c*) suggestions of fibrils, as in Fig. 1, or often, granular areas devoid of organelles. Advancing anterior, pseudopodial regions were characterized by (*a*) smooth plasmalemma, (*b*) much less dense ground plasma than in uroid regions, (*c*) no suggestion of fibril structures. No attempt was made here to correlate the presence or absence of a hyaline zone, but many sections of advancing areas with the characteristics involving the anterior half of the amebae were examined. These differences between regions were more striking in cells returning to streaming after treatment with Alcian blue. Fig. 4 shows a uroid region after such treatment; compare this figure with Fig. 5 which shows an advancing pseudopodial region after similar treatment. Note the dense appearance of surface coat (*G*) in Fig. 4 compared to Fig. 1, due to the adsorbed dye.

As noted before (Nachmias, 1964), extensive networks of filaments beneath the cell membrane and parallel arrangements of filaments and fibrils deeper in the cytoplasm were commonly found in cells treated with Alcian blue and fixed with the same osmium tetroxide that revealed only traces of filaments in dye-untreated tail regions, as in Fig. 1. In Alcian blue-treated cells, thick fibrils were also found after alkaline osmium tetroxide or glutaraldehyde fixation, as were areas of dense granular material in which organelles were excluded (see Hayward, 1963).

Pellet

Fig. 6 shows the appearance of the fibrils in the pellet fraction. The *x* is placed just above a row of fibrils cut in cross-section. Note dense interiors of fibrils. Arrows point to material that is adhering to, or is part of, the fibril. Groups or single fibrils were found in this heterogeneous pellet with high frequency. They have a maximum length of 0.5 μ and a diameter of 12–15 $m\mu$. They appear identical with thick fibrils seen deep in the cytoplasm of some sectioned cells after Alcian blue treatment (Fig. 7). Thin filaments were not seen in the pellets. A second spin of the 50,000 *g* supernatant at 70,000 *g* for 15 min in one experiment resulted in a pellet that appeared to have higher concentration of fibrils than the 50,000 *g* pellet.

Negative Staining

Frequently, only positively stained thick filaments were found in the supernatant from the 50,000 *g* pellet (Fig. 8). Arrows point to two thick fibrils. Various results were obtained: sometimes distinct, separated fibrils 0.5 μ long, in numbers up to 170–180 per grid space (200 mesh), were found; in other cases, tangled meshworks of fibrils were seen, usually when excess material was present.

In some preparations negatively stained fibrils were found (Fig. 9). In this example, there were only about 10 fibrils per grid space. In some cases, as in one of the fibrils in Fig. 9 (arrow), finer strands \sim 4–6 $m\mu$ in diameter seem to emerge from the body of the fibrils; when traced into the body of the fibrils, the strands appear coiled or twisted. There is also some material adhering to the surface of the fibrils. Similar material is also seen clearly in the pellet fraction (Fig. 6), but is not seen in the sectioned cytoplasm (Fig. 7).

DISCUSSION

These results clearly demonstrate the existence of two types of filaments in the cytoplasm of *Chaos chaos*. While previous results were obtained only on dye-treated cells fixed with a single fixative, in this study filaments were found, after glutaraldehyde fixation, in intact cells and in a pellet fraction after homogenization of cells, and with either osmium tetroxide or glutaraldehyde fixation after dye pretreatment of cells. The fact that thick fibrils can be found in a pellet fraction and in the supernatant by negative staining shows that

these fibrils are stable enough to be spun out without fixation, at least in the presence of 10 mM ATP. The present findings are in general agreement with the results of others on *A. proteus* (Komnick and Wohlfarth-Bottermann, 1965; Wohlfarth-Bottermann and Komnick, 1966; Bhowmick and Wohlfarth-Bottermann, 1966; Wolpert et al., 1964) and *Trichamoeba villosa* (Bhowmick, 1967) and confirm the previous report on *Chaos chaos* (Nachmias, 1964).

The question arises whether the thick fibrils represent a separate entity. Two observations support the suggestion (Nachmias, 1963) that the thick type of fibril represents some kind of aggregation of thinner filaments. First, in sectioned material images are often seen that are thicker and denser than nearby thin filaments but are less well formed than thick fibrils. Such images appear intermediate in character between clear-cut thin filaments and thick fibrils. Second, negative staining of the supernatant from the 50,000 g pellet fraction shows thin filaments emerging from thick fibrils. These two observations accord with other results (Wolpert et al., 1964). It should be pointed out, however, that the conclusion that thick fibrils may be formed from thin filaments does not rule out the presence of some other component as well.

Before further work can be done on the interesting questions of the localization of the filaments and fibrils during streaming and under different physiological conditions, better fixation is required. Glutaraldehyde would be the fixative of choice since it is a rational fixative which has proved

essential for the preservation of microtubules. Unfortunately, and interestingly, it produces a variable amount of distortion in almost all cells and, even when distortion is minimal, streaming continues for 2-3 min during fixation (Table I). A variety of attempts was made to reduce the fixation time, including addition of ethylenediaminetetraacetic acid, Mg²⁺ ions, or dimethyl sulfoxide to the fixative, or altering the strength, temperature, or pH of the glutaraldehyde or the rate of addition of the fixative to the specimens. None of these methods produced any noticeable improvement in terms of shortening the duration of fixation or preventing the occurrence of cell distortion (Table I). Very recently, we found that as little as 0.05% osmium tetroxide added to the glutaraldehyde directly before use prevents prolonged streaming and cell distortion. It remains to be seen whether this modified fixative will prove to be useful for further study of these interesting cytoplasmic filaments.

A brief report of some of this work has appeared (1966. *J. Cell Biol.* 31:154A.).

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